Fungal Biotransformation of Morphine Alkaloids.

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Abstract

The purpose of the study was to determine the ability of certain fungi to biotransform morphine alkaloids into medicinally relevant intermediates. Fungal strains screened for their ability to affect biotransformation of morphine alkaloids include *Cunninghamella echinulata, Helicostylum piriforme, Pycnoporus sanguinea, Pycnoporus cinnabarina, Curvularia lunata and Sporotrichum sulfurescens.* The research demonstrated that *Cunninghamella echinulata* *N*-demethylated thebaine, hydrocodone, codeine, oripavine and oxycodone into corresponding nor-compounds in varying yields.

The study further focused on the characterization of the enzyme responsible for the biotransformation of thebaine into northebaine by *Cunninghamella echinulata.* The study clearly showed that incubation of the fungal culture with thebaine over a period of 48 hours was required to activate the biotransformation process.

The biotransformation studies with [^{14}C] labeled thebaine showed that *N*-demethylation by *Cunninghamella echinulata* does not involve *O*-demethylation followed by methyl group transfer as suggested in previous studies.

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List of Abbreviations

ATCC	American type culture collection
BIA	benzyl-isoquinoline alkaloids
CMI (IMI)	Commonwealth Mycological Institute
CMYG	corn meal yeast glucose
CNS	central nervous system
COR	codeinone reductase
DCM	dichloromethane
DRR	dehydroreticuline reductase
DRS	dehydroreticuline synthase
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GCS	glucose corn steep solution
HCl	hydrochloric acid
HPAA	hydroxyl phenyl acetaldehyde
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
INCB	International Narcotics Central Board
IR	infra red
mp	melting point
MS	mass spectrometry
NaOH	sodium hydroxide
NCS	norcoclaurine synthase
NRRL	Northern regional research laboratory (National centre for agricultural utilization research)
NMR	nuclear magnetic resonance

NADPH	nicotine adenine dinucleotide phosphate reduced
NRM	nutrient rich medium
ppm	parts per million
RPM	revolutions per minute
SAT	salutaridinol acetyl transferase
SOR	salutarine oxidoreductase
STS	salutaridine synthase
SAM	*S*- adenosyl methionine
SAH	*S*- adenosyl homocysteine
TLC	thin layer chromatography
TYDC	tyrosine decarboxylase
UNODC	United Nations Office on Drugs and Crime
UPLC-MS	ultra performance liquid chromatography mass spectrometry

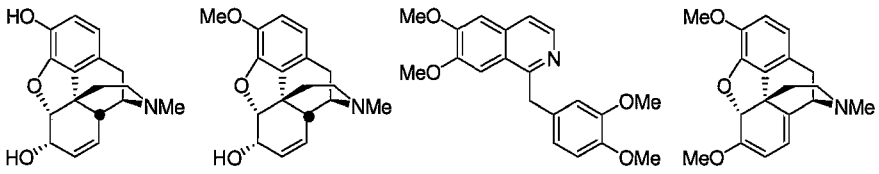
1. Introduction

Opium has been fascinating mankind since the days of recorded history. Morphine alkaloids are extensively used as medicines for the treatment of pain and are equally abused. The estimated total amount of raw opium produced worldwide in 2007 was 8200 tons.[1] Raw opium contains varying amounts of morphine (**1**), codeine (**2**), narcotine, papaverine (**3**) and thebaine (**4**).[2] The most famous among opiates, morphine, was isolated by Friedrich Wilhelm Sertuerner[3] in 1805. Even today opium poppy remains the only commercially viable source of morphine alkaloids.

Over the last two decades, a major goal of the Hudlicky group has been to develop a practical synthetic route for morphine alkaloids, capable of competing with the natural supply. A genuinely practical synthesis would ensure a constant supply of morphine (**1**) to satisfy the world's demand for this valuable drug. Moreover, it would eliminate the world's dependency on the production of natural morphine in countries where there is political instability such as Afghanistan, the country with the highest opium production in the world.

The Hudlicky group's interest in morphine alkaloids is not limited to their total synthesis. In cooperation with Noramco Inc., a subsidiary of Johnson & Johnson, we became interested in the conversion of naturally occurring morphine alkaloids into more valuable derivatives utilizing economic and environmentally benign transformations both chemical and biological. An ideal starting material for the preparation of semi-synthetic analgesics is thebaine (**4**), a minor constituent in the latex of opium poppy. Tasmanian Alkaloids, in partnership with Noramco Inc.

developed a genetically engineered poppy plant, the latex of which contains up to 30% thebaine.[4]

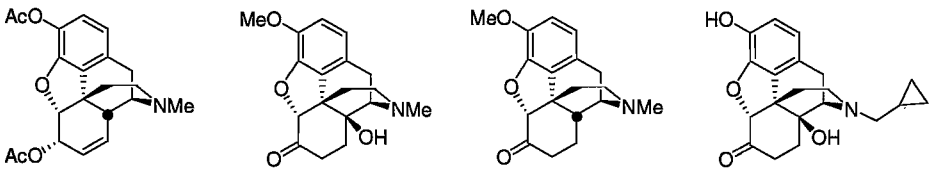


morphine **1** codeine **2** papaverine **3** thebaine **4**

Figure 1. Morphine alkaloids

Semi-synthetic drugs derived from opiates have affected society for more than 100 years. The first attempt to make a non-addictive morphine substitute was made by the English chemist Charles Robert Albert Wright[5]. He synthesized the semi-synthetic opiate diacetylmorphine (**5**), better known as heroin, in 1898. Years later, the even more addictive properties of heroin (**5**) compared to morphine were realized and the drug had to be taken out of the market in 1910.

Shortly after the mass production of heroin was stopped, two semi-synthetic opioids, oxycodone (**6**) and hydrocodone (**6**) were discovered[6]. Both derivatives are still marketed throughout the world as they possess less addictive and euphoric properties than morphine. Naltrexone[7], (**8**) an opioid antagonist, is also a semi-synthetic drug derived from thebaine and is used for the treatment of alcohol and opioid dependence. The manufacturing of semi-synthetic opioids represents a prospering market.



diacetylmorphine **5** | oxycodone **6** | hydrocodone **7** | naltrexone **8**

Figure 2. Semi-synthetic opioids

The current thesis will discuss the production of some of the intermediates for the synthesis of semi-synthetic opiates by microbial transformations. *N*-demethylation of morphine alkaloids are the key step in the synthesis of semi-synthetic opioids such as naltrexone (**8**). Chemical dealkylation has the disadvantage of the involvement of expensive and sometimes highly toxic reagents[8-10]. Microorganisms can be used as an alternative means of preparing *N*-dealkylated drug intermediates. We envisioned screening six fungal strains for their ability to *N*-demethylate six different morphine alkaloids.

Hydroxylation at the C-14 position is an important oxidative procedure in the commercial production of morphine-derived antagonists such as naltrexone, naloxone, nalbuphine and other medicinally significant compounds. The ability of fungi to perform C-14 hydroxylation of morphine alkaloids has been demonstrated in the early 1960s by Iizuka.[11-13] Out of the six fungal strains tested two transformed thebaine to 14- hydroxycodeine in varying yields. But the ability of these fungi for C-14 hydroxylation had been demonstrated earlier.[11, 14]

Convinced by the versatility of *Cunninghamella echinulata* to transform morphine alkaloids, attempts to isolate and characterize the enzyme responsible for *N*-demethylation were initiated. The vision of Prof. Tomas Hudlicky was to successfully

isolate the enzyme and clone the appropriate gene for genetic engineering of this step in *E. coli and* enable a commercial production of *N*-demethylated morphinans by fermentation. Apart from the characterization of fungal cultures capable of biotransforming morphinan alkaloids, this thesis describes the difficulties in developing an enzyme assay to evaluate the activity of demethylase in cell free extracts of *Cunninghamella echinulata.*

2. Historical

2.1. Opium poppy and morphine alkaloids

Papaver somniferum, the opium poppy is one of mankind's oldest medicinal plants and it is the only commercial source of the narcotic analgesics morphine and codeine. Along with these two morphinans, opium poppy produces approximately eighty alkaloids belonging to various classes of tetrahydrobenzylisoquinoline alkaloids.[15]

Raw opium is the air-dried milky exudate or latex that is obtained by incising the unripe capsules of the opium poppy and has been the traditional source of poppy alkaloids. In recent times, the entire plant tops (poppy straw) are harvested, dried and then extracted for their alkaloid content in the pharmaceutical industry.[16] Extremely large profits can be made from smuggling relatively small amounts of opium and converting it to heroin.[17] The use of bulkier poppy straw in pharmaceutical production reduces the chances of using it for illicit opium trade as poppy straw is difficult to smuggle, compared to opium. The main producer of medicinal opium is India, whilst poppy straw is cultivated in Turkey, Russia and Australia.[16] Opium destined for the black market originates form the Golden Triangle (Myanmar, Laos and Thailand), the Golden Crescent (Iran, Pakistan and Afghanistan) and Mexico.[16] Despite millennia of compulsive use and abuse the opiates are still unrivalled as analgesics and derivatives of opium components continue to be indispensable in modern therapeutics.

Legal production of opiate raw materials, both latex-derived and poppy straw derived, is restricted to assigned countries. The International Narcotics Control Board

(INCB) is responsible for monitoring the legal supply of, and demand for, opiates in addition to maintaining an acceptable global "balance" that accommodates the medicinal needs of human populations. The United Nations Office on Drugs and Crime (UNODC) compiles annual reports documenting the illicit cultivation of opium poppy.[1]

Currently, only India exports raw opium, although other Asian countries China, North Korea and Japan are also entitled to its production. Australia, specifically the island of Tasmania, supplies a large proportion of the world's thebaine.[16, 4] Thebaine is a starting material in the manufacture of several semi-synthetic opiates, including oxycodone, oxymorphone, etorphine, and buprenorphine. Additionally, thebaine is the starting material for the synthesis of naloxone, naltrexone, nalorphine, and nalbuphine, some of which are used to treat opiate poisoning and opium addiction.[17] Thebaine was mainly obtained as a byproduct from opium. The development of the poppy mutant[4] known as *top1* (for 'thebaine oripavine poppy 1'), in 1998, which accumulates thebaine and oripavine, facilitated an agriculturally viable supply of thebaine and is a crop that carries little risk of diversion for illicit purposes.

2.1.1. History of morphine alkaloids

Opium has its origins at the onset of human society and its use almost certainly predates recorded history. Opium is undoubtedly the first drug to be discovered by mankind[18]. Being naturally occurring, it almost certainly predates the discovery of alcohol which requires knowledge of the fermentation process required for its production.

The preserved remains of cultivated poppy seeds and pods have been discovered in the sites of Neolithic pile-dwelling villages in Switzerland[18, 16] of 4th millennium BC. It has long been suggested that the knowledge of opium spread from Egypt through Asia Minor to the rest of the Old World, but the Swiss discoveries cast this theory into doubt. It is possible that the discovery and use of opium originated in the eastern reaches of Europe, in the Balkans or around the Black Sea and spread south and west from there.

Around 3400 BC, the opium poppy was being cultivated in the Tigris-Euphrates river systems of lower Mesopotamia. The Sumerians, the world's first civilization and agriculturists, used the ideograms[16, 18, 19] *hul* and *gil* for the poppy, which means the *'joy plant'*. The ancient Sumerians recognized the euphoric properties of opium. Their invention of writing spread gradually to other societies and it is from them that the Egyptians probably learned about opium. By the end of the second millennium BC knowledge of opium was widespread throughout Europe, the Middle East and North Africa. References to opium poppy and its preparations appear in the Talmud and the Bible. Roman culture incorporated Greek knowledge of opium poppy. The routine supplying of opium to Roman soldiers[19] and the extensive growth of the Roman Empire facilitated the widespread cultivation and supply of the plant for food, medicine, and oil.

The earliest discovery of raw opium itself comes from Egypt where a sample was found in the tomb of Cha, dating to the 15th century BC. At around the same time, the Egyptian city of *Thebes* was so famous that Egyptian opium[16, 19] was known as *Thebic* opium and the alkaloid, *thebaine*, obtains its name from the city. In the sixth

century BC, a Persian text mentioned opium. Opium was called "*afiun*" by the Persians[18b].In the third century BC, the Greek philosopher, Theophrastus referred to the sap of the pod of poppy plant as "*opion*" and the poppy juice, "*meconion*", obtained by crushing the entire plant.[18] It has been postulated that the word "*opium*" was derived from the Greek word "*opion*".

Crude opium has been used since antiquity as an analgesic, sleep inducer (narcotic) and for the treatment of coughs. In the Therapeutic Papyrus[18, 20] of Thebes (Papyrus of Ebers), dated 1552 BC and in other sources such as the Veterinary and Gynecological Papyri from Kahun, dated between 2160 and 1788 BC, opium is prominently listed with other natural remedies and drugs. In the Papyrus of Ebers opium is included in 700 remedies.[18] Hippocrates (460-357 BC), the father of Medicine, suggested drinking hypnotic meconion (white poppy juice) mixed with nettle seeds to cure leucorrhea and 'uterine suffocation'. He prescribed preparations of opium poppy as a hypnotic, narcotic, stypic and cathartic. He also acknowledged the nutritive properties of the seeds.[19]

Opium was used in the early Egyptian, Greek and Arabic cultures primarily for its constipating effect in the treatment of diarrhea. Later, opium's sleep inducing properties were noted by writers such as Homer, Virgil, and Ovid.[19] In countries where alcohol was prohibited because of religious beliefs, opium was commonly used as an intoxicant. The socioeconomic importance of opium was described in the travel accounts of ancient Portuguese and Dutch mariners who reached the Far East in search of opium and spices, especially pepper.[21] The nearly bankrupt British East India Company stabilized its financial situation with opium exports to China. This made

India the main producer of opium. The British forced the Chinese to permit the opium trade and consumption that eventually triggered the first (1838-1842) and the second (1856-1860) "Opium Wars" between Great Britain and China.[21, 22] The Chinese lost the war and therefore their efforts to suppress the sale and use of opium failed miserably.

The invention of *laudanum*, an alcoholic herbal preparation of opium, by the Swiss physician Paracelsus established opium as a premium pain killer in Europe. Paregoric, or camphorated opium tincture, was used in the treatment of severe diarrhea and dysentery. In Dover's powder, powdered opium[17] was combined with powdered ipecacuanha or ipecac to give a popular sedative and diaphoretic to take at the onset of colds and influenza. One major drawback of administering crude opium preparation was that the content of the active ingredients was unknown to the physician.

2.1.2. Biosynthesis of morphine alkaloids in opium poppy

Papaver somniferum is an annual herb with a growth cycle of approximately 120 days. In general, poppies require fertile, free draining soil which is not overly acidic. The best growing climate is temperate, warm with low humidity and not too much rainfall during early growth[18]. The poppy is a 'long day' photosensitive plant. Blossoming requires a growth period of long days and short nights, preferably with direct sunlight at least twelve hours daily.

Agents such as nitrofene and chlortoluron are frequently used to control weeds, while treatments with aminophos-methyl, furathiocarb, dimethoate, diazinon or malathion can provide protection against insects during stem emergence periods. Seed-treatment prior to sowing, application of sulfur- or copper-containing preparations, and

optimal harvesting times can protect opium poppy from harmful fungi. Virus infected plants are usually burnt to restrict the spread of the infection.[16]

Under ideal conditions, the main blossom appears around the ninetieth day from germination. The plant produces large solitary flowers, of white, pink, crimson or weakly purple color. Maturation of the seed pod/capsule/bulb/poppy head occurs about 110-150 days after sowing. One pod may produce over 1000 seeds.[18]

Opium is only produced during a ten to twelve day period when the pod is ripening. Once it has reached its maturity, the alkaloids are no longer made and are broken down in time. Opium is harvested by tapping individual pods. The ripening capsules, which are just changing color from blue-green to yellow, are carefully incised with a knife to open the latex tubes.[17] The initially white, milky latex quickly oozes out, but rapidly turns brown by oxidation and coagulates. This raw opium is then removed early the following morning (farmers hope that more opium oozes out in overnight time from lanced capsule), being scrapped off and molded into balls or blocks. Typically these are wrapped in poppy leaves and shade dried because raw opium contains a high percentage of water.[23] Fresh opium is pale to dark brown and plastic. A pod will continue to secrete opium for some days (may be tapped ~6 times). The average opium yield is 80 milligrams per pod (a hectare of poppies providing between 8 and 15 kilograms of raw opium).

In 1805, a German pharmacist, Friedrich Wilhelm Serturner, isolated morphine[3] (Morpheus, the Greek God of Dreams) in its pure form from raw opium. Serturner identified morphine as a potent active component of opium. Besides morphine (**1**), which accounts for 10-16% of weight, raw opium contains varying

amounts of codeine (**2**) 1-3%, narcotine (**10**) 1-7%, papaverine (**3**) 0.8-1%, thebaine (**4**) 0.5-2% and traces of oripavine (**9**).

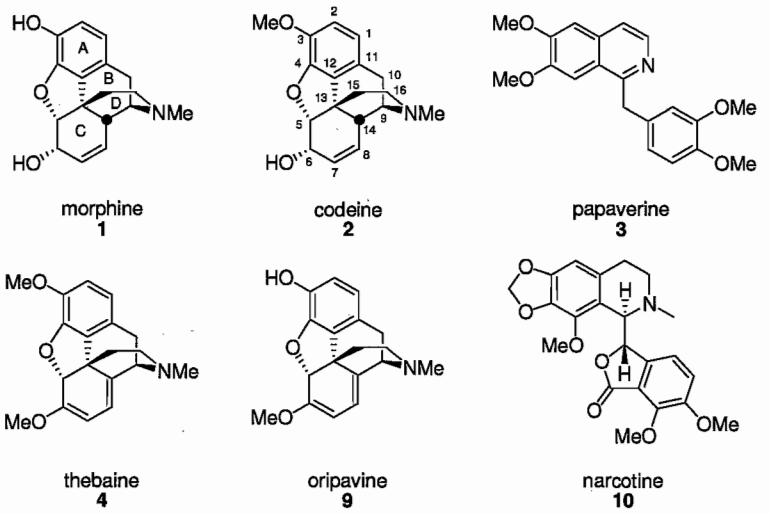


Figure 3. Naturally occurring morphine alkaloids

Pathways for morphine biosynthesis

The enzymatic synthesis of morphine has been almost completely elucidated, many of the encoding genes have been cloned, and the corresponding recombinant enzymes have been characterized. Benzylisoquinoline alkaloid (BIA) biosynthesis begins with the conversion of tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) (**13**). Tyrosine decarboxylase (TYDC) converts tyrosine to tyramine and dopa (**11**) to dopamine (**12**) (Figure 4). Dopamine and 4- HPAA are condensed by norcoclaurie sythease (NCS) to yield (*S*)-norcoclaurine (**14**), the central precursor to all benzylisoquinoline alkaloids in plants.[24] (*S*)-norcoclaurine is converted to (*S*)-reticuline (**18**) in a four step enzymatic reaction involving three methyl transferases and a P450 hydroxylase[25, 26] (CYP80B1) (Figure 4).

(*S*)-reticuline is a key branch point intermediate in benzylisoquinoline alkaloid biosynthesis and a variety of subsequent enzymatic reactions determine the structural type of alkaloid produced.[16]

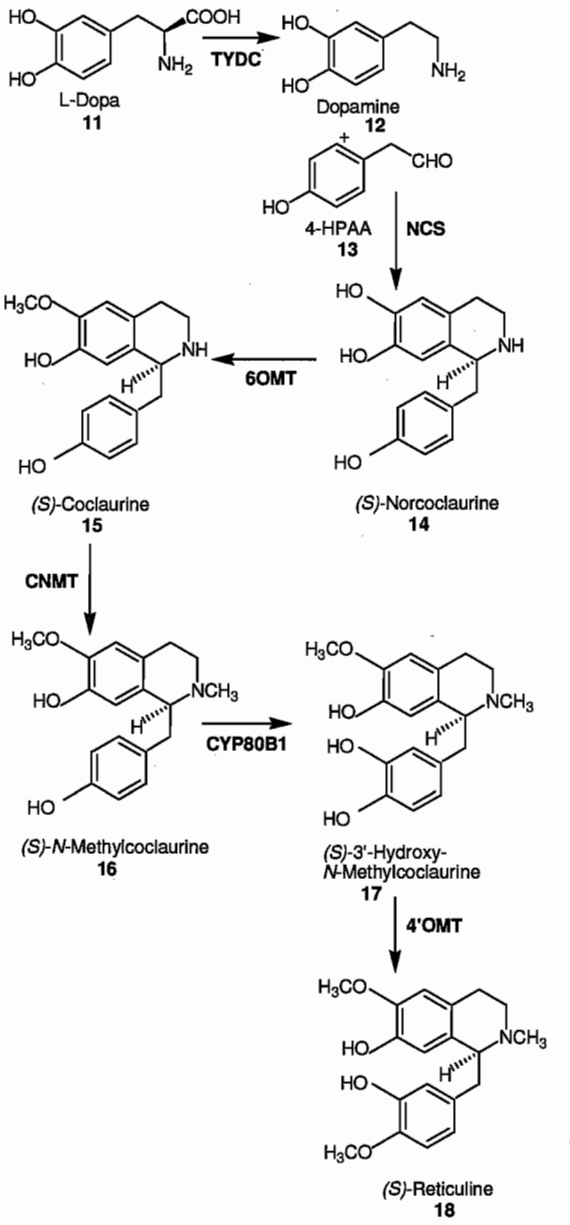


Figure 4. Biosynthesis of *(S)*-Reticuline

Conversion of (*S*)-reticuline (**18**) to its (*R*)-epimer (**20**) is the first committed step in morphine alkaloid biosynthesis. The oxidation and subsequent reduction of (*S*)-reticuline (**18**) to (*R*)-reticuline (**20**), via 1,2-dehydroreticuline (**19**), is catalyzed by two enzymes 1,2-dehydroreticuline synthase (DRS) and 1,2-dehydroreticuline reductase[27] (DRR). Intra-molecular carbon-carbon phenol coupling of (*R*)-reticuline by the P450-dependent enzyme salutaridine synthase (STS) results in the formation of salutaridine (**21**).[28] The cytosolic enzyme, salutaridine NADPH 7-oxidoreductase (SOR) reduces salutaridine (**21**) to (7S)-salutaridinol (**22**).[29] Acetyl coenzyme A:salutaridinol-7-*O*-acetyltransferase (SAT) catalyzes the conversion of salutaridinol (**22**) to salutaridinol-7-*O*-acetate (**23**), which spontaneously produces thebaine (**4**).[30]

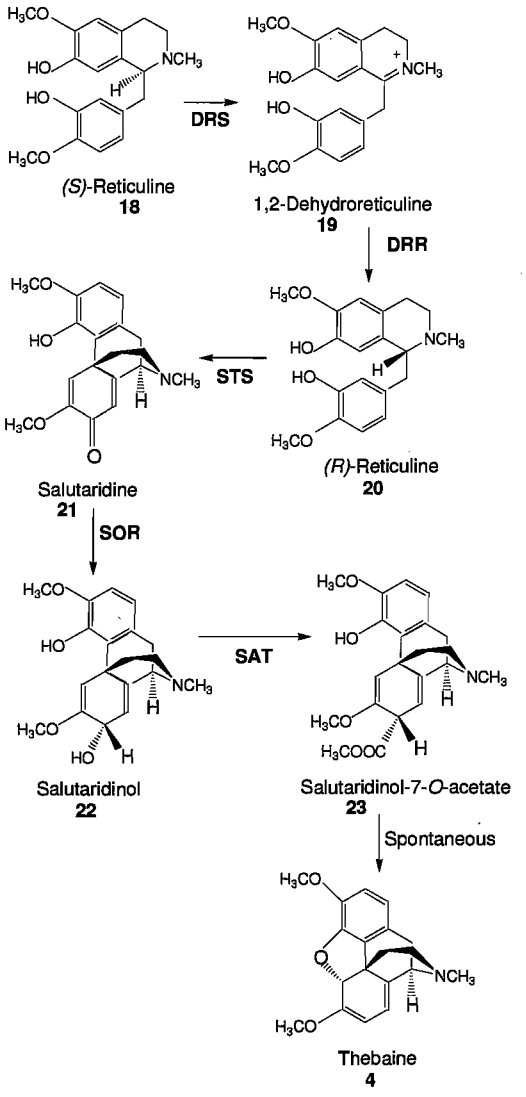


Figure 5. Biosynthesis of thebaine from *(S)*-reticuline

The pathway from thebaine (**4**) to morphine (**1**) involves two demethylation steps and proceeds by two alternative routes. The demethylation of thebaine results in the formation of either neopinone (**24**) or oripavine (**9**) depending on the position (6 or 3) of demethylation. Neopinone (**24**) spontaneously forms codeinone (**25**), which is reduced by the NADPH-dependent enzyme codeinone reductase (COR) to form codeine (**2**).[31] Demethylation of codeine (**2**) yields morphine (**1**). An alternative route[32]

for morphine biosynthesis involves the production of morphinone (**26**) from oripavine (**9**) by demethylation, followed by COR-catalyzed reduction of morphinone (**26**) yielding morphine (**1**). This alternative biosynthetic pathway was further proven by the preparation of a poppy mutant known as *top1* (for 'thebaine oripavine poppy 1').[4] The seeds of *Papaver somniferum* (commercial poppy cultivar) were treated with a mutagen and the progeny plants were screened for mutants. The mutant *top1* was found to accumulate thebaine (**4**) and oripavine (**9**) but not morphine (**1**) or codeine (**2**). Feeding experiments with radioactive intermediates confirmed that there was a block in both arms of the bifurcated pathway at thebaine (**4**) and oripavine (**9**). The authors postulated a defect in the enzyme thebaine demethylase, which is responsible for the 6-*O*-demethylation of both thebaine (**4**) and oripavine (**9**). The development of the *top1* mutant not only contributed to the understanding of the biosynthetic pathway of morphine alkaloids, but also provided an agriculturally viable supply of thebaine (**4**) and oripavine (**9**), which are employed in the manufacturing of the vast majority of semi-synthetic opioids. The crop of *top 1* mutant carried little risk of diversion for illicit purposes due to the absence of morphine.

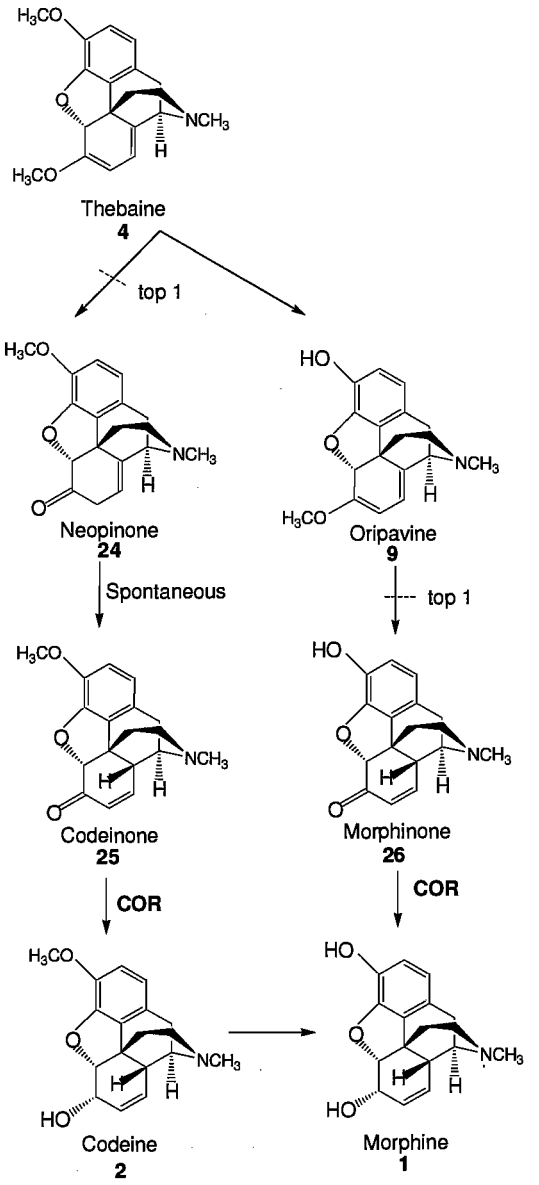


Figure 6. Biosynthesis of morphine from thebaine

Recently, the cell type-specific localization of benzylisoquinoline alkaloid (BIA) biosynthesis was determined.[33] Overall the process from gene expression through alkaloid accumulation requires three specialized cell types, companion cells, vascular phloem sieve elements and laticifers. Laticifers, the specialized cells in the phloem that contain the latex, serve as the site for alkaloid accumulation, not

biosynthesis, in opium poppy. Further sub-cellular compartmentalization of BIA enzymes occurs due to the cytotoxicity[34] of the pathway intermediates and products. The non-cytosolic enzymes in morphinan biosynthetic pathways are localized to the endoplasmic reticulum (ER) or ER-derived endomembranes.[24]

2.1.3. Pharmacology of morphine analgesics

The pharmacological attributes of opium poppy (*Papaver somniferum* L.) have been appreciated even before the dawn of civilization. Even today, opium poppy remains the only source for the analgesic morphine, the antitussive codeine and the muscle relaxants papaverine and noscapine. Thebaine, another natural product of opium poppy is used as a starting material for the production of oxycodone and other semi-synthetic opiates.

Morphine is the major pain relieving agent obtained from opium. Unfortunately, morphine has the same potential for abuse as opium. Attempts to develop safer and more efficient drugs providing analgesia with reduced side effects and abuse potential led to the synthesis of heroin (diacetylmorphine) in 1874. Ironically, heroin is more addictive than morphine. The first pure opioid antagonist naloxone was produced in the 1940s.[22]

Opioid receptors

The important pharmacological differences shown by opioid analgesics are derived from their complex interactions with three opioid receptor types, μ, δ and κ. Based on the activities of stereo isomers, the concept of pharmacologically relevant receptors for opioids was first elaborated by British medicinal chemists Arnold Beckett and Casy in 1954.[35] Later, the concept of different modes of interaction of morphine

and other analgesics with opioid receptors was suggested by Portoghese in 1965.[36] In 1971, Goldstein proposed that radio labeled compounds might be used to demonstrate the existence of these receptors and to characterize them.[37] In 1973, when radioligands with high specific activities were available, three different groups,[38] independently but simultaneously, showed that there are stereo-specific opioid binding sites in mammalian brain.

By the mid 1960s, it was becoming clear that the actions of opioid agonists, antagonists, and mixed agonist/antagonist could be explained best by their variable interactions with multiple opioid receptors[36] (Portughese 1965). In 1976, the group of Martin[39] provided the first convincing evidence for this concept. The authors proposed three types of opioid receptors, named after the drugs used in these studies *viz.* mu (μ, for morphine, which induces analgesia, miosis, bradycardia, hypothermia and indifference to environmental stimuli), kappa (κ, for ketocyclazocine, which induces miosis, general sedation and depression of flexor reflexes) and sigma (σ, for SKF 10,047 or N-allylnormetazocine, which induces mydriasis, increased respiration, tachycardia and delirium).

The most important neurotransmitter system involved in nociception is composed of the endogenous opioid peptides (endomorphins, enkephalins, dynorphins and endorphins) and their corresponding receptors (μ, κ, δ) which are expressed within specialized neurons. In 1975, Kosterlitz[40] and coworkers discovered enkephalins. This event led to the suspicion that morphine mimicked endogenous analgesic compounds and it was not a natural opioid receptor ligand. Further studies proved that the opioid receptors are part of the endogenous opioid system.

The knowledge that opioid receptors are present in the periphery along with the Central Nervous System (CNS) led to the development of functional models of opioid action. Preparations of the isolated ileum of the guinea pig and of the vas deferens from mouse, rat, rabbit and hamster have been used in pharmacological assays to assess the agonist/antagonist properties of opioids. Based on their observations of the action of morphine and naloxone[41] on guinea pig ileum and mouse vas deferens Kosterlitz proposed a fourth type of opioid receptor named delta *(δ,* deferens *)*. A few years later, Mannalack[42] and co-workers showed that the σ receptor is non-opioid in nature. Thus, there are three main types[43, 44] of pharmacologically defined opioid receptors, μ, κ and δ. All the receptors have been cloned confirming their existence and all have binding and functional properties consistent with their identities.[45, 46] Pain relief effects are mediated by all three receptors but to different degrees.

Studies conducted on the cloned μ, κ and δ opioid receptors demonstrate that the amino acid sequences of the three receptors are 65% homologous and all of them interact with heterotrimeric G-proteins[47]. The G-protein coupled receptor superfamily includes numerous neurotransmitter and hormonal receptors that transmit signals via second messengers (cyclic Adenosine monophosphate) or ion channels (K^+). Alterations in the levels of cyclic AMP during long-term morphine treatments are associated with a number of cellular changes, including the development of tolerance and physical dependence.

μ receptors are located at the supraspinal sites of the body which includes medial thalamus and brain stem. Activation of μ receptors in these areas lead to analgesic, respiratory depressant, miotic (pinpoint pupils), euphoric and physical

dependence properties of morphine and its related drugs. κ receptors are located principally within the dorsal horn of the spinal cord. κ receptors produce analgesia by depressing the initial relay site of pain transmission. κ specific drugs produce miosis and sedation but not euphoria, physical dependence, or respiratory depression. These drugs are known to produce aversive effects in animals and dysphoria in humans. Delta receptors are poorly delineated. The activity of each drug at each type of receptor indicates whether it activates (agonistic action) or inhibits (antagonistic action) the receptor function.[19]

Classification of opioid analgesics by analgesic properties

Opioid analgesics can be classified using the knowledge of opioid receptors. According to its function of activation or inhibition of each type of receptor, each drug can be categorized as a pure agonist, a pure antagonist or a mixed agonist/antagonist.

Agonist

Substances which bind to a receptor and induce changes in the cell characteristic of the natural ligand for the receptor are called agonists. Pure agonists exhibit a high binding affinity for (and exert high activity with) μ receptors and they also have affinity for kappa receptors. Morphine, the prototype of a pure opioid agonist, stimulates opioid receptor of the mu type. As a result, analgesia, relaxed euphoria, sedation, a sense of tranquility, reduced apprehension and concern, respiratory depression, suppression of the cough reflex, and papillary constriction occurs. Examples of naturally occurring opioid agonists are morphine and codeine. Semi-synthetic opiates heroin (diacetylmorphine), meperidine, methadone, oxycodone, hydrocodone, oxymorphone, hydromorphone and fentanyl are also agonists.[19, 20]

Antagonist

Pure antagonists bind to opioid receptors with varying affinity, but they do not exert agonist activity at any receptor. Two clinically available drugs, naloxone (Narcan) and naltrexone (Trexan) are pure opioid antagonists[19]. Both can compete with the mu agonist for the receptor, precipitating withdrawal in an opioid dependent person reversing any analgesia caused by the agonist. Naloxone is neither analgesic nor subject to abuse. Naloxone is used to reverse the respiratory depression that follows acute narcotic intoxication (overdoses). Naloxone is not absorbed from intestine and its duration of action is only 15 to 30 minutes. Naltrexone is the first orally absorbed pure narcotic antagonist. Naltrexone has a long duration of action, necessitating only a single daily dose. Naltrexone is used for the treatment of heroin dependency as well as alcoholism.[19] Currently methylnaltrexone, a derivative of naltrexone is a preferred antagonist for treating opioid dependence because of its restricted ability to cross the blood brain barrier.[48] Methylnaltrexone reverses the undesired side effects of opioids (that are mediated by the peripheral opioid receptors) like respiratory depression or withdrawal symptoms, without affecting the central opioid receptors.[49]

Mixed Agonist/Antagonist

A mixed agonist/antagonist exhibits modest agonistic activity at some receptors and antagonist effect at another. In general, these drugs are weak mu agonists. Their analgesic effectiveness results from stimulation of κ receptors. Examples of mixed agonist/antagonist are pentazocine, butorphanol, nalbuphine, buprenorphine and dezocine.[50] Being weak agonist or antagonist of the mu opioid receptor, these drugs do not have the potential for respiratory depression or physical dependence.

2.1.4. Semi-synthetic opiates

A vast range of semi-synthetic or totally synthetic morphine-like derivatives are produced, which are referred to as opioids. Many of these opioids are analgesics but less habit forming and some are antagonistic in action. More than 90% of morphine is used to produce other derivatives. For example, antitussive **codeine** and **pholcodine** are derived from morphine. Analgesics with less side effects **dihydrocodeine** and **hydromorphone** also are produced from morphine. **Heroin** or **diamorphine** is the first semi-synthetic opioid from morphine which is highly addictive, analgesic and hypnotic. *N*-alkyl derivative of morphine include **nalorphine**, a mixed agonist-antagonist.[17]

In addition to morphine, thebaine is now utilized for the semi-synthesis of useful new drugs. Analgesics **oxycodone** and **oxymorphone** are derived from thebaine. Another analgesic **etorphine** a derivative of thebaine is too powerful for human use. Etorphine is used to sedate large animals like elephants and rhinos. Etorphine is approximately 5000- 10000 times more potent than morphine.[17] **Buprenorphine** and **nalbuphine** are examples of mixed agonist-antagonist produced semi-synthetically from thebaine. Buprenorphine is *N*-alkyl derivative of etorphine while nalbuphine is *N*-alkyl derivative of oxymorphone. Antagonists in use **naloxone** and **naltrexone** are *N*-alkyl derivatives of oxymorphone/ oxycodone which are originally derived from thebaine. Thebaine may also be transformed into codeine in about 75% yield.

2.2. Microbial biotransformations

Microorganisms had been the friend and foe of human beings since the dawn of civilizations. Without even being aware of the existence of microbes man was using them in the production of food and beverages. Before 6000 BC the Sumerians and Babylonians were practicing the brewing of beer. References to wine making can be found in the Book of Genesis and the Egyptians used yeast for the leavening of bread.[51, 52]

In the field of industrial microbiology, microorganisms are put to work to make a product by a process called fermentation. Typically, for a cell, fermentation is a way of getting energy by breaking down complex organic molecules to simpler ones without using oxygen. In the pharmaceutical or biotechnology industry, fermentation is any large scale cultivation of microbes occurring with or without air. In recent years, microbial fermentations have been revolutionized by the application of genetically engineered organisms. Currently antibiotics produced by fermentation include bacitracin, streptomycin, neomycin, erythromycin, tetracycline, penicillin and chloramphenicol. Genetically engineered organisms produce highly valuable hormones like human insulin, human growth hormone and bovine growth hormone by fermentation.[53]

In the course of time it was discovered that microorganisms can modify certain compounds by enzymatic catalysis. Nowadays this process is called biotransformation. The essential difference between fermentation and biotransformation is that there are several catalytic steps between the substrate and the product in fermentation while there is only one or two in biotransformation. Also, the chemical structures of the

substrate and product resemble one another in biotransformation, but not necessarily in fermentation. An example of biotransformation is vinegar production which dates back to around 2000 BC. Vinegar production is perhaps the oldest and best known example of microbial oxidation.[51]

2.2.1. Fungal biotransformations

Fungi play important ecological and economic roles. Being saprophytic, they continue the cycle of nutrients through ecosystems. Fungi possess a battery of enzymes which are very versatile in the degradation of xenobiotics. White rot fungi are generally recognized for their ability to oxidize pollutants. *Cunninghamella elegans*[54] has been used as a microbial model for mammalian xenobiotic metabolism. This ability to degrade xenobiotics makes fungi the best choice for testing the biotransformation of various toxic chemicals and drugs. In order to obtain derivatives which are potentially useful for detailed pharmacological studies fungal biotransformations of existing drugs are also carried out.[55-59].

Fungal biotransformations may permit stereospecific conversion of readily available starting materials to valuable products. Fungal biotransformation products can be utilized in the chemoenzymatic synthesis of pharmaceuticals. Biotransformation of cycloalkenones by fungi *Curvularia lunata*[60] (NRRL 2380) produces synthetically important chiral lactones in high yields. Fungi are useful for mild, selective oxidations of lupane substrates at positions C-3, C-7, C-15, C-25 and C-30. Endophytic fungi, for example, *Aspergillus fumigatus*[61] can perform stereo-selective biotransformation (sulfoxidation) of thioridazine, a phenothiazine neuroleptic drug. (R)-phenylacetyl carbinol is the chiral precursor in the manufacture of pharmaceuticals ephedrine and

pseudoephedrine. Filamentous fungi *Rhizopus javanicus*[62] can biotransform benzaldehyde into (R)-phenylacetyl carbinol.

A well known example of fungal biotransformation is the detoxification of chlorophenols by white rot fungi. Methylation of phenolic hydroxyl groups or reductive dehalogenation is the biotransformation approach of cholorophenols by *Phanerochaete chrysosporium*[63]. Chlorophenolic hydroxyl group can also be glycosylated by fungi *Trametes versicolor* and *Pycnoporus cinnabarinus.*

Some fungi can even biotransform antifungal agents. The triphenylmethane dye, malachite green, is a widely used antifungal agent in the fish farming industry. Malachite green was enzymatically reduced to leucomalachite green by the filamentous fungi *Cunninghamella elegans*[64] (ATCC 36112). Malachite green was also converted to *N*-demethylated and *N*-oxidized metabolites, including primary and secondary aryl amines.

Apart from biotransformation of pharmaceuticals and pollutants, fungi also play a major role in the biotransformation of terpenoids (also known as isoprenoids) in fragrance industry. Many flavors and fragrance compounds contributing to a wide range of pleasant scents are biosynthetically derived from isoprene units. An example of terpenoid biotransformation is the conversion of limonene to carveol and carvone by *Penicillium digitatum.*[65]

2.2.2. Fungal biotransformation of morphine alkaloids

In the early 1960s, microbial transformations of morphine alkaloids were studied extensively. The prime motive behind biotransformation was to produce more effective analgesics from naturally available compounds. Japanese research groups

first reported the biotransformation of morphine alkaloids by fungi. Iizuka[66] and coworkers (1960) described the conversion of thebaine to 14-hydroxycodeine and 14-hydroxycodeinone by the basidiomycete, *Trametes sanguinea.* Yamada[67, 68] (1962) showed that *Trametes sanguinea* transformed codeinone to codeine, 14-hydroxycodeine and 14-hydroxycodeinone. In 1969, the fungus *Trametes cinnabarina* was also found to convert thebaine to 14-hydroxycodeine, 14-hydroxycodeinone and 14-hydroxycodeinone *N*-oxide by Groger[70]. Stabler[73] demonstrated the oxidation of morphine to 2,2' bimorphine (pseudomorphine) by fungus *Cylindrocarpon didymium.* Other than fungal transformations, several groups have reported bacterial transformations of morphine alkaloids.

2.2.3. *N*-demethylation of morphine alkaloids

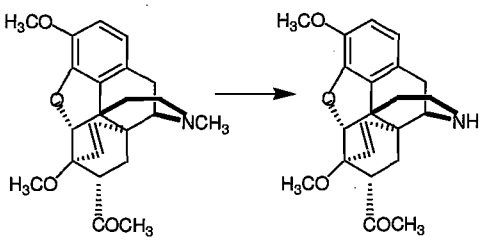
N-dealkylation of pharmaceutically important molecules is an important step in drug synthesis and manufacture. The *N*-substituent of many drug molecules has considerable pharmacological importance. Variations of the *N*-substitution may cause substantial variation in pharmacological activity[75, 76]. The design of new drugs often require removal of the *N*-alkyl function usually methyl from the parent drug followed by *N*-acylation or *N*-arylation of the *nor*- intermediate.

Direct chemical *N*-dealkylation reactions are difficult and hazardous to achieve. Moreover, chemical dealkylation has the disadvantages of variable product yields and the involvement of expensive and highly toxic reagents.[77-79]

Microorganisms can be used as an alternative means of preparing *N*-dealkylated drug intermediates with the following benefits. Microbial transformation may give rise to increased and more consistent yields. Microbial enzymatic reactions

are highly specific. Microorganisms may mediate some reactions which are not chemically possible. Above all, microbial transformations are usually conducted under mild conditions, in aqueous media, with low cost raw materials as growth media.[80]

A number of groups have reported the biotransformation capabilities of genus *Cunninghamella*. As early as 1968, Mitscher reported the *N*- demethylation of 6, 14-*endo*-ethenotetrahydrothebaine by *Cunninghamella echinulata* (NRRL-A-11498)[69]. In 1984, Sewell[71] reported the biotransformation of codeine to nor-codiene by a number of species of *Cunninghamella* namely *C. bainieri* C43, *C. bertholletiae* C1, *C. blakesleeana* CMI 53585, *C. echinulata* IMI 199884, *C. echinulata* C7 and *C. echinulata* NRRL 3655, in yields of approximately 10%. Madyastha[72] in 1994, found that the fungus *Mucor piriformis*, transformed thebaine to *nor*-thebaine in ~77% yield. *Mucor piriformis* also produced isomeric thebaine *N*-oxides. Abel demonstrated the synthesis of potential buprenorphine intermediates by selective microbial *N* and *O* demethylations in 2002.[74] Of the eleven species of *Cunninghamella* employed in this synthesis *C. echincelata* (NRRL 2384) gave 39% yield of the *N*-demethylated compound.



27 28

Figure 7: *N*-demethylation of 6,14- *endo*-ethenotetrahydrothebaine

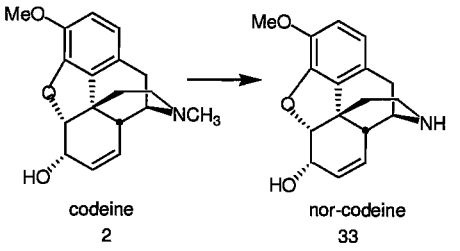


Figure 8: *N*-demethylation of codeine

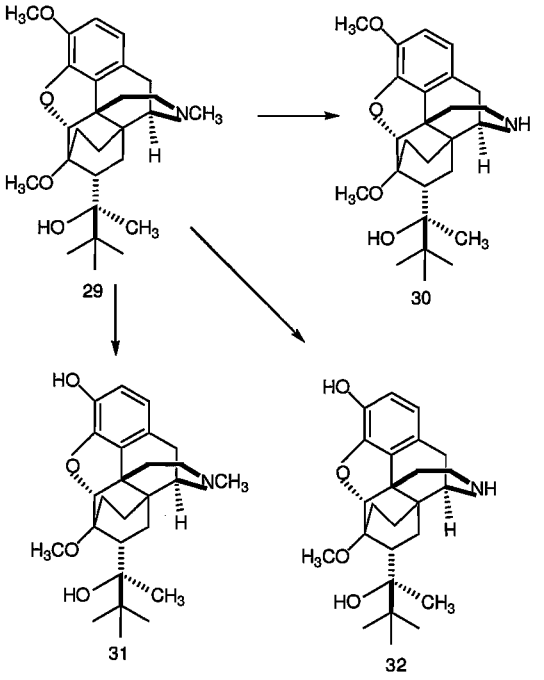


Figure 9: Synthesis of buprenorphine intermediates

by microbial *N*-, *O*, or *N*- and *O*-demethylation

3. Discussion

3.1. Biotransformation of morphine alkaloids

3.1.1. *N*-demethylation of morphine alkaloids by *Cunninghamella echinulata*

The presented research discusses the potential of the fungi *Cunninghamella echinulata* (ATCC 9244) to *N*- demethylate a variety of morphine alkaloids. The strain *Cunninghamella echinulata* (ATCC 9244) used in the present study *N*-demethylated thebaine, hydrocodone, codeine, oxycodone and oripavine. The maximum accumulation of the demethylated product was seen when thebaine was fed to the cultures. There was a significant difference in the conversion rates of morphine alkaloids depending on the morphology of the fungi. When Erlenmeyer flasks were used for biotransformation, the fungi produced thick mycelium which aggregate together to form a single huge biomass. The conversion rate was low with this morphology. Therefore, baffled flasks were used for biotransformation in which the fungi grew as small pellets. The surface area of fungal mycelium exposed to the medium it grew, containing the alkaloid, increased considerably in the case of pellets which led to better biotransformation rates.

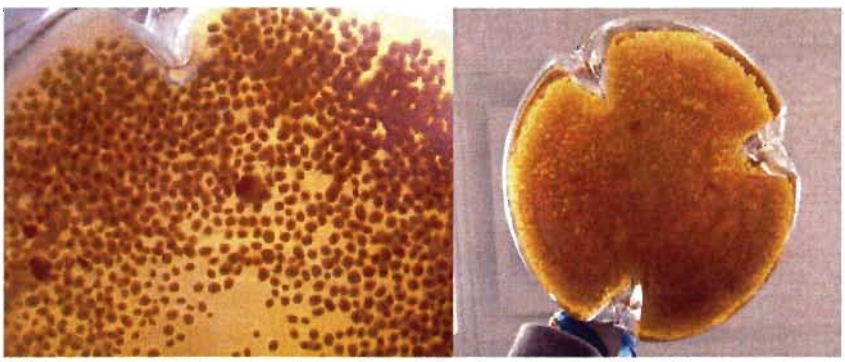


Figure 10: Growth of *Cunninghamella echinulata* in a baffled flask as small pellets

The yield of *nor*-thebaine product from thebaine was 35-50%. Hydrocodone, codiene and oripavine were converted to corresponding *N*-demethylated products *nor*-hydrocodone, *nor*-codeine and *nor*-oripavine in 10% yields. The yield of *nor*-oxycodone from oxycodone was 15%. This is the first account of microbial *N*-demethylation of the alkaloids hydrocodone, oxycodone and oripavine. There was no biotransformation when either morphine or oxymorphone was used as the substrate. The starting material was recovered in both cases. Morphine and oxymorphone lack a methoxy group at both C3 and C6 position. This could be the reason for no biotransformation. It may be necessary to have a methoxy group at the C3 or C6 position, which may help the molecule to fit into the active site of the enzyme responsible for demethylation.

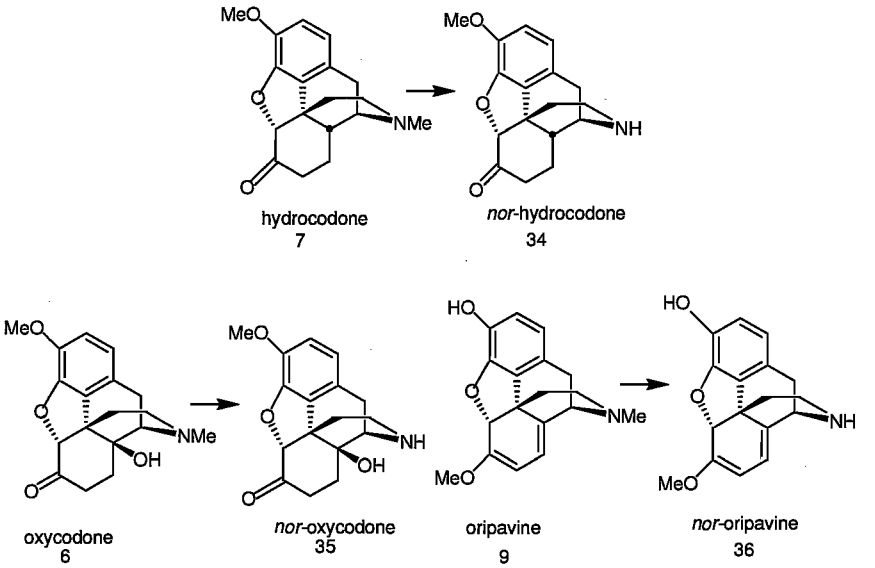
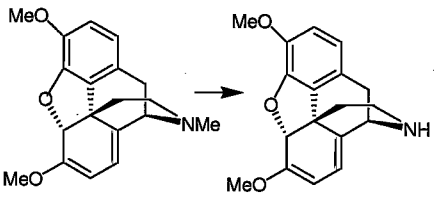


Figure 11: *N*-demethylation of morphine alkaloids by *Cunninghamella echinulata*

N*-demethylation of thebaine by *Thamnostylum piriforme

The versatility of the genus *Cunninghamella* in *N*- demethylation of morphine alkaloids have been patented by Carnell and coworkers in 2007[81]. The only fungal transformation reported to date involving the *N*- demethylation of thebaine, was demonstrated by Madyastha K.M. in 2000 using *Mucor piriformis*[82]. The fungus *Mucor piriformis* was isolated in India and is not available for purchase.



thebaine
4

nor-thebaine
37

Figure 12: *N*-demethylation of thebaine

Thamnostylum (Helicostylum) piriforme (ATCC 8992) is known for 14α-hydroxylations of steroids[83]. A screening of fungal strains was done for 14-hydroxylation of morphine alkaloids. Surprisingly, instead of 14- hydroxylation *Helicostylum N*- demethylated thebaine in 39% yield. Other than steroids *Helicostylum* has only been demonstrated to transform strychnine[84]. Inspired by the yield of *N*-demethylation of thebaine, other morphine alkaloids were tested for biotransformation. Interestingly, hydrocodone and oxycodone was reduced at the C6 position with 40% and 10-15% yield respectively. When codeine, oripavine and morphine were used as the substrate there was no biotransformation.

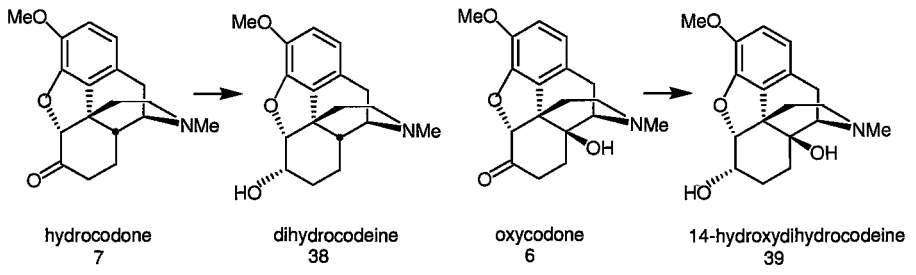


Figure 13: C6 reduction of hydrocodone and oxycodone

3.1.2. C14- hydroxylation of morphine alkaloids

Researchers envisaged the discovery of a magic drug which would counteract the deleterious effects of opium alkaloids on the human body, without loss of analgesic properties[85]. Attempts have been made to find products that are able to antagonize or neutralize the addictive properties of morphine alkaloids. Hydroxylation at the C-14 position is an important oxidative procedure in the commercial production of morphine- derived antagonists such as naltrexone, nalbuphine and other medicinally significant compounds.

Microorganism's ability of C14 hydroxylation of morphine alkaloids has been demonstrated in the early 1960s by Iizuka and coworkers.[66-68] In fact, this group reported the first microbial transformation within the class of morphine alkaloids using the fungi *Trametes sanguinea*. C-14 hydroxylation is a crucial step in the synthesis of semi-synthetic opiates like oxycodone. World-wide marketed oxycodone has less addictive and less euphoric properties than morphine.

In an attempt to find novel fungal strains capable of C-14 hydroxylation five fungal species namely *Trametes (Pycnoporus) sanguinea* (ATCC 14622), *Trametes (Pycnoporus) cinnabarina* (ATCC 14623), *Sporotrichum sulfurescens (Beauveria bassiana*, ATCC 7159), *Helicostylum (Thamnostylum) piriforme* (ATCC 8992) and

Curvularia lunata (ATCC 12017) were screened for biotransformation of morphine alkaloids.

T. sanguinea and T. cinnabarina could accomplish C-14 hydroxylation of thebaine as reported before. But when hydrocodone was fed to these fungal cultures it produced C6 reduction. C6 reduction was also observed with *T.sanguinea* when oxycodone was used as the substrate whereas there was no biotransformation when *T.cinnabarina* was fed with oxycodone. There was no biotransformation when codeine, oripavine or morphine was added to both the cultures. 9-40% yield of 14-hydroxycodiene was detected in the biotransformation of thebaine by *T. sanguinea* whereas only 15% product accumulated when *T. cinnabarina* was used as the biocatalyst.

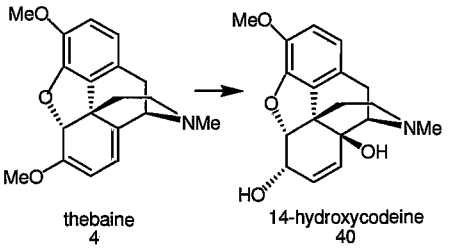


Figure 14: C14-hydroxylation of thebaine

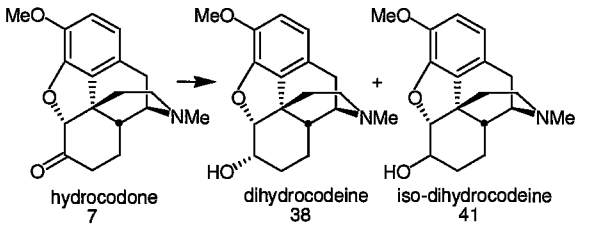


Figure 15: C6-reduction of hydrocodone in the ratio 5:4

Sporotrichum sulfurescens is known for the hydroxylation of monocyclic alcohols,[86] 1-benzoyl-trans-decahydroquinoline[87] and *N*-acyl-1-adamantamines.[88] Based on this data the fungus was assayed for morphine alkaloid biotransformation. No product formation was observed in any of the alkaloid tested (thebaine, hydrocodone, oxycodone, codeine, oripavine and morphine). However, two unidentified metabolites accumulated in the media in all the biotransformation experiments. These metabolites were present in the media even in the absence of morphine alkaloids ruling out the possibility of stress induced production.

Microbiological hydroxylation of 12a-deoxytetracycline by *Curvularia lunata* was established in 1959 by Holmlund.[89] *Curvularia* is also widely known for its 11β-hydroxylation of steroids.[90] Therefore, the fungal cultures were treated with various morphine alkaloids. None of the morphinans tested underwent C14 hydroxylation in the presence of *Curvularia*. However, it is worth noting that C6 reduction was carried out on both hydrocodone and oxycodone with 40% yield of dihydrocodeine and 63% yield of 14-hydroxydihydrocodeine. Oxycodone biotransformation resulted in the formation of both isomers of 14-hydroxydihydrocodeine. Future optimization of C6 reduction by *Curvularia* may lead to a commercially viable process in producing intermediates for the synthesis of semi-synthetic opiates. *Curvularia* also produced two metabolites (Figure 16) in all the biotransformation procedures irrespective of the presence or absence of morphine alkaloids.

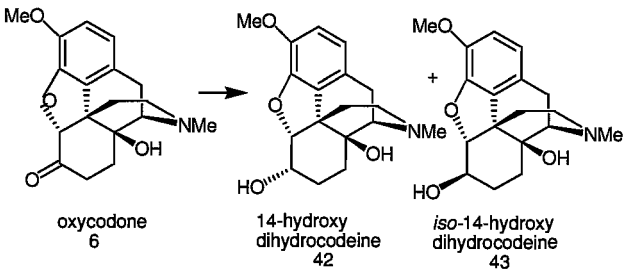


Figure 16: C6 reduction of oxycodone in the ratio 3:1 by *Curvularia lunata*

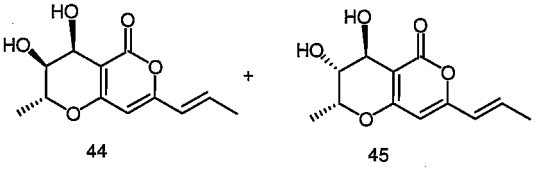


Figure 17: Metabolites produced by *Curvularia lunata*

Overall, morphine was untouched by all the fungal species. Only *Cunninghamella* biotransformed codeine and oripavine, all the other fungal species did not use these as substrates. It can be concluded that the methoxy group at C3 position plays a significant role in biotransformations.

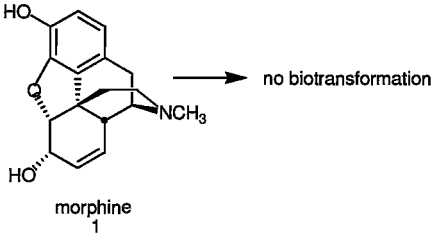


Figure 18: None of the fungal strains tested transformed morphine

Table 1: Biotransformation of morphine alkaloids by various fungi

Substrate	*Cunninghamella echinulata*	*Helicostylum piriforme*	*Trametes sanguinea*	*Trametes cinnabarina*	*Sporotrichum sulfurescens*	*Curvularia lunata*
Thebaine	*N*-demethylation 35-50% yield	*N*-demethylation 40% yield	14-hydroxy-codeine 9-40% yield	14-hydroxy-codeine 15% yield	No biotrans-formation	No biotrans-formation
Hydrocodone	*N*-demethylation 10% yield	C6 reduction 40% yield	C6 reduction 12% yield	C6 reduction 35% yield	No biotrans-formation	C6 reduction 40% yield
Oxycodone	*N*-demethylation 15% yield	C6 reduction 10-15% yield	C6 reduction 19% yield	No biotrans-formation	No biotrans-formation	C6 reduction 63% yield
Codeine	*N*-demethylation 10% yield	No biotrans-formation	No biotrans-formation	No biotrans-formation	No biotrans-formation	No biotrans-formation
Oripavine	*N*-demethylation 9-21% yield	No biotrans-formation	No biotrans-formation	No biotrans-formation	No biotrans-formation	Not tested
Morphine	No biotrans-formation	No biotrans-formation	No biotrans-formation	Not tested	No biotrans-formation	No biotrans-formation

3.2. Characterization of the enzyme involved in demethylation of morphine alkaloids from *Cunninghamella echinulata*

The Hudlicky group widely appreciates the potential of biocatalysis. The starting materials for the total synthesis of analgesic morphine, antitussive codeine, antiviral balanol and anticancer 7-deoxypancreastatin, are dihydyroxylated arenes produced by fermentation with recombinant *E.coli* JM109[104]. Since a highly efficient chemical demethylation procedure for morphine alkaloids remains unavailable, this has prompted the more detailed characterization of *Cunninghamella echinulata* biotransformation system for *N*-demethylation of thebaine in order to identify the enzyme mechanism involved. This study was conducted in collaboration with **Professor Vincenzo De Luca**, Department of Biology, **Brock University** and the pharmaceutical company **Noramco Inc.**, a subsidiary of **Johnson & Johnson.**

3.2.1 Biotransformation of thebaine into northebaine by cultures of *Cunninghamella echinulata* (ATCC 9244)

Cunninghamella echinulata (ATCC 9244) fungal cells were transferred to individual flasks containing fresh medium to grow for 2 days after which Thebaine (0.5 mg/ml) was added for biotransformation studies. Individual flasks were harvested at two day intervals by separating the media from the fungal biomass by filtration. Analysis of the media and the fungal biomass showed that Thebaine as well as biotransformation products were mostly found in the medium (~80% of the alkaloids were recovered from the medium). Detailed analysis of alkaloid profiles in the media showed that while no biotransformation of thebaine takes place in the first 2 (48 h)

days of culture, most of the biotransformation of thebaine into northebaine takes place 48 to 92 h (day 4) after the addition of thebaine (Figure 19).

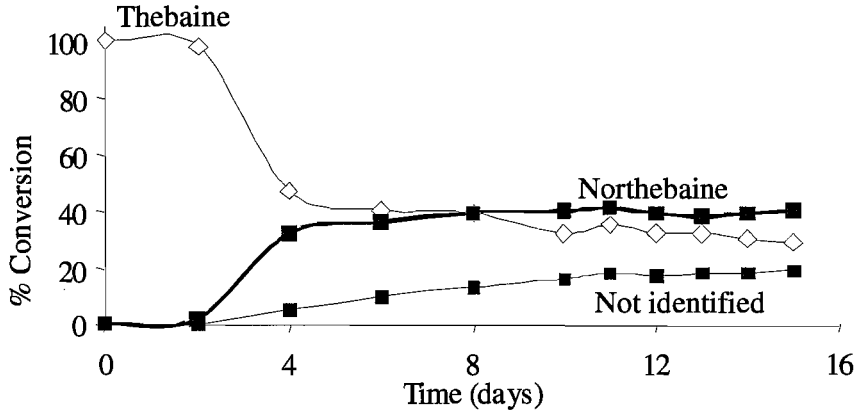


Figure 19: Conversion of thebaine to northebaine by *Cunninghamella echinulata* over a period of fifteen days.

By day 4 of biotransformation, more than 50 % of the thebaine has been depleted with almost 40 % of this alkaloid being converted to northebaine (Figure 19). Beyond day 4 of biotransformation, the level of thebaine conversion slowed down almost completely and the level of northebaine accumulation leveled off. Between days 4 to 15 of biotransformation a new thebaine biotransformation product identified as thebaine-*N*-oxide, which accounted for approximately 20 % of the total thebaine derivatives was found in the media between days 10 to 15 of the fermentation (Figure 19). This experiment was repeated at least 3 times, some slight variations in the results obtained, but in all cases the biotransformation pattern over the first six days remained very similar. During the 2 to 4 day period of biotransformation, the fungal culture converted an average of 2.6 mg/hour of thebaine into northebaine.

In order to more precisely determine the kinetics of thebaine conversion into northebaine, the experiment was repeated and fungal cultures were harvested more frequently between days 2 to 5 for analysis of the biotransformation. The results clearly indicate that the conversion of thebaine into northebaine required an incubation of two days before it was initiated (Figure 20).

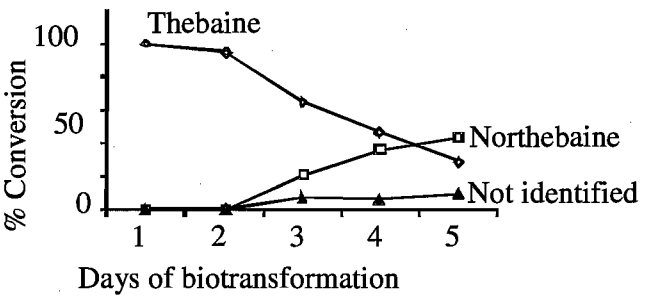


Figure 20: Biotransformation of thebaine over a period of 5 days. Thebaine was added after two days of fungal growth. Total fungal growth period was seven days.

Since thebaine could activate this biotransformation reaction in 2 day old cultures (Figures 19 and 20), it was investigated if older fungal cultures could also respond to thebaine treatment in a similar manner. When thebaine was added to 5 day old fungal cultures, they responded by progressively converting thebaine into northebaine over the next 5 days (Figure 21). Close to 50 % of the thebaine had been converted into northebaine after 5 days of biotransformation as found for the same experiment performed with 2 day old cultures. While similar results with small variations in the kinetics were obtained when the experiment was repeated with 7 and 8 day old cultures (data not shown), the yield in older cultures of northebaine production was only 10 %.

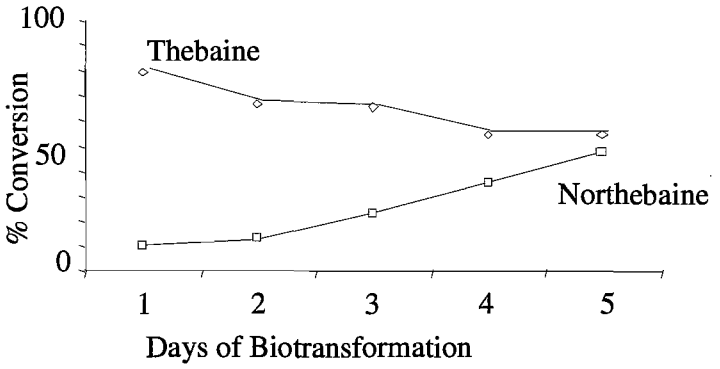


Figure 21: Biotransformation of thebaine over a period of 5 days. Thebaine was added after five days of fungal growth. Total fungal growth period was ten days.

The data (Figures 19-21) clearly suggested that fungal cultures at different stages of the growth cycle will transform thebaine into northebaine and that the component(s) required for this biotransformation are not present constitutively within the culture, but might be induced over a period of 48 hours by the addition of thebaine.

3.2.2 Pretreatment of cultures of *Cunninghamella echinulata* with a low dose of thebaine induces the components required for biotransformation of thebaine into northebaine.

Fungal cells were grown in the presence or absence of a low concentration of thebaine (0.05 mg/ml) for 9 days after which 0.5 mg/ml of thebaine was added to each culture to test the biotransformation efficiency of the culture. Media harvested for 6 consecutive days was extracted for alkaloids and was analyzed for northebaine production (Figure 22).

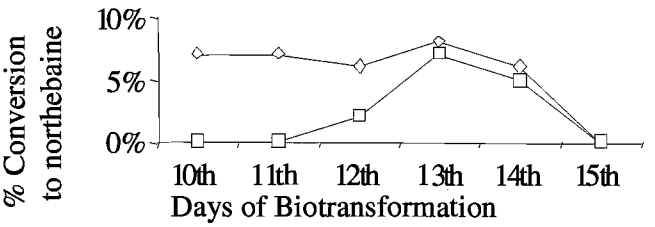


Figure 22: Biotransformation of thebaine by *Cunninghamella echinulata* cultures.

Cells pre-induced with low levels of thebaine already produced maximum amounts of northebaine within 24 h of concentrated thebaine addition (Figure 22). In contrast little or no biotransformation of thebaine occurred in the first 48 h of culture in uninduced cells inoculated with thebaine (0.5 mg/ml). The 48 h lag was followed with a progressive increase in the rate of conversion that reached similar levels found in pre-induced cells by day 13 of the biotransformation (Figure 22). However the levels of northebaine declined to zero with further growth of both induced and uninduced cells, suggesting that the northebaine was being converted to other compounds with incubation time. The lag observed in the formation of northebaine in non-induced cultures and the rapid induction of thebaine biotransformation ability by pre-incubating fungal cells with low levels of thebaine (Figure 19) clearly suggests that biotransformation capability is induced in this system. The experiments provide strong evidence that the search for enzymes involved in this biotransformation should be conducted with 2 day old fungal cells that have been treated for at least further 48 h with low levels of thebaine (Figures 19-22).

3.3 Identification of enzymes involved in the biotransformation of thebaine into northebaine in *Cunninghamella echinulata* cultures.

3.3.1 Characteristics of enzymes involved in demethylation reactions:

In general, demethylase enzymes are usually Cytochrome P450[71, 98], flavin or 2-oxoglutarate[105] dependent oxidases. In one rare case, the demethylase was a cobalamin dependent methyltransferase which transferred the methyl group from an *N*- to a free hydroxyl group on the molecule[99]. Cytochrome P450 and flavin dependent enzymes are membrane associated, while 2-oxoglutarate dependent enzymes are found in the soluble fraction of cell free extracts. Cytochrome P450 enzymes require reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor, whereas flavin dependent enzymes need flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as coenzymes. 2-oxoglutarate dependent enzymes require the cofactors α-ketoglutarate (2-oxoglutarate), ascorbic acid and iron. If the demethylase is a methyl transferase, then it can either be membrane associated or soluble. The cofactors necessary for methyl transferases are S-adenosyl methionine (SAM or AdoMet) and S-adenosyl Homocysteine (SAH or AdoHys).

3.3.2 Putative mechanisms for *N*-demethylation of morphine alkaloids

N-demethylation of morphine alkaloids can occur directly[98] or via the formation of an *N*-oxide intermediate (Figure 23). In addition, Abel[99] reported that *N*-demethylation of morphinans in the *Cunninghamella* could involve a cobalamin methyl transferase. They used a deuterium labeled thebaine derivative for feeding experiments using *Cunninghamella echinulata* (NRRL 1384) fungal culture. They

reported that *O*- demethylation occurs first, which is followed by a methyl transfer from *N*- to *O*- position (Figure 23).

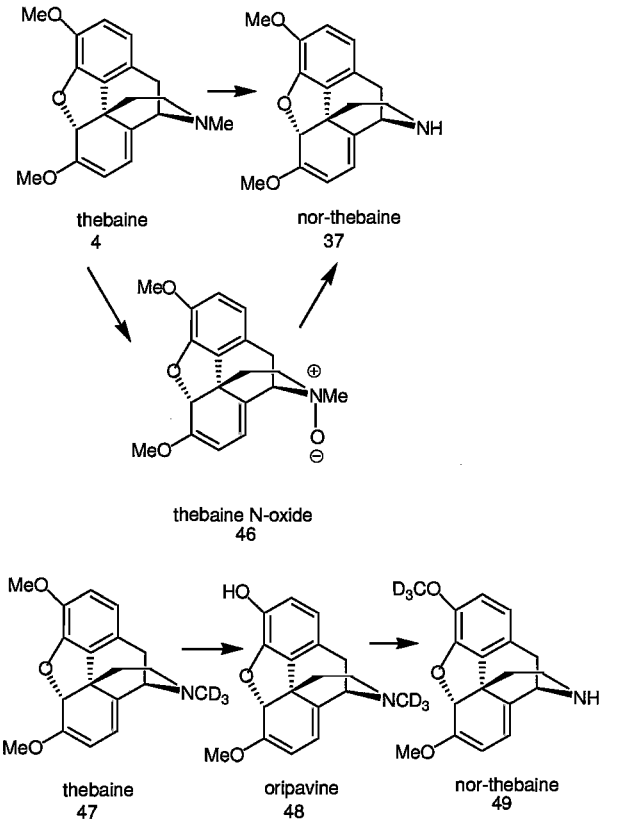


Figure 23: Putative mechanism for *N*- demethylation of thebaine

3.3.3 *In vitro* enzyme assay development for conversion of thebaine into northebaine.

A number of different approaches were attempted to develop an enzyme assay for conversion of thebaine into northebaine. Initial studies were conducted with cells that had been induced by adding 0.2 mg thebaine/ml to the cell culture as described in Materials and Methods. Unfortunately these extracts were contaminated with residual thebaine and northebaine that made impossible to differentiate *de novo* synthesized

northebaine from that already present in the extract due to biotransformation. This led to replacing the thebaine with the same concentration of oripavine which was able to activate the fungal cell demethylation system. Cell free extracts from these induced cultures did not interfere with the detection of any northebaine would be produced in enzyme assays since TLC or by UPLC-MS (Figure 24) could resolve this product from oripavine and nororipavine contaminants which.

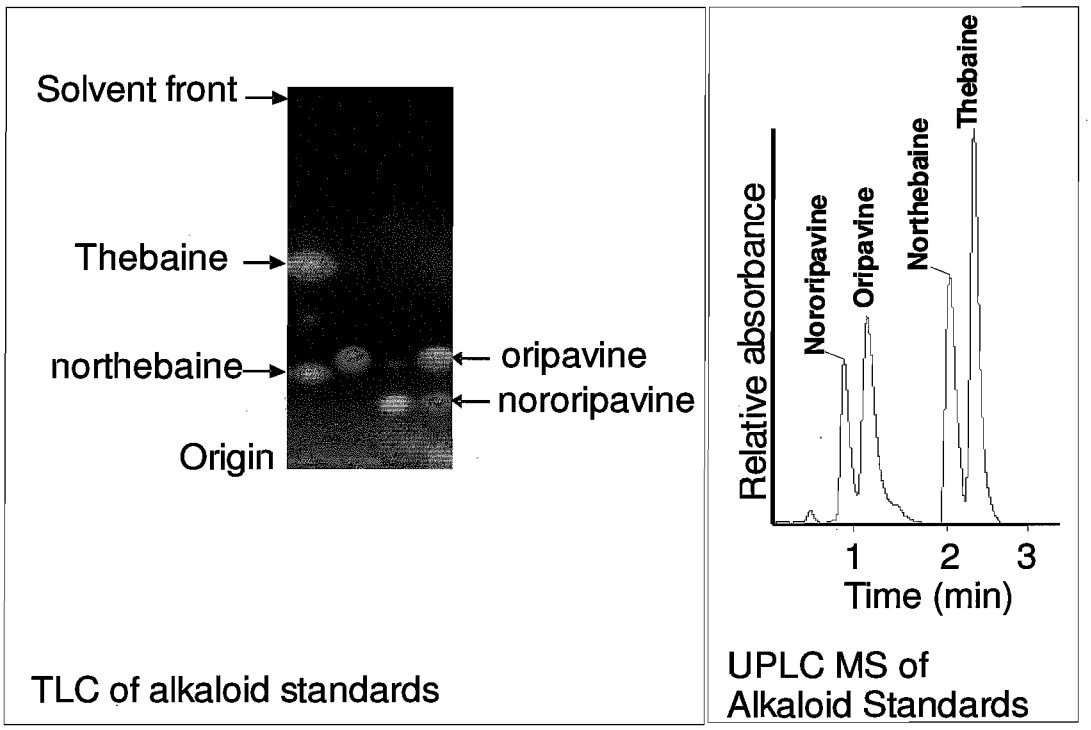


Figure 24: Separation of morphinan alkaloid standards by TLC and UPLC-MS.

3.3.4 Cell free extracts assayed under different conditions do not convert thebaine into northebaine.

3.3.4.1 Extraction of Frozen cells

Frozen cells were extracted as described in Method 1 (See Materials and Methods). This crude extract was assayed (Appendix 1, Table 1) to test for the presence of a Cytochrome P450 or 2-Oxoglutarate dependent dioxygenase involved in northebaine formation. Under these conditions, no reaction products were detected.

3.3.4.2 Extraction of fresh cells

Since storage of cells at -80 °C could inactivate the components involved in conversion of thebaine into northebaine, fungal cells were grown in the presence of oripavine to activate the components involved in conversion of thebaine to northebaine. Cells were tested for their biotransformation competence by harvesting media and analyzing the alkaloid profiles by TLC at different points in the growth cycle. Since maximal conversion appeared to occur 3 days after cells were transferred to fresh oripavine containing growth medium, cells were harvested for enzyme assays by filtration and were extracted according to Method 2 in Materials and Methods. Enzyme assays were performed as described in Materials and Methods to test for the presence of a cytochrome P450, 2-oxoglutarate dependent and flavin dependent enzymes that might be involved in northebaine formation. The enzyme assays from extracts of fresh cells were inactive irrespective of the cofactors used in the assay (Appendix 1, Table 2).

The experiment with fresh cells was repeated with the same assays as described in Appendix 1, Table 3). Additional assays were conducted in the presence of AdoMet and AdoCys to test if the mechanism involving a cobalamin dependent methyltransferase[98] might be involved in the conversion of thebaine into northebaine. As in the previous experiment, none of the assays performed catalyzed this cell free conversion (Appendix 1, Table 3).

3.3.4.3 Extraction of fresh cell and enzyme assay using the protocol of Gibson.[98]

In 1984, Gibson first reported the *N*-demethylation of codeine[98] by cell-free extracts of *Cunninghamella bainieri*. The mechanism suggested involved an *N*-demethylation of tertiary amines to form either an intermediate *N*-oxide or a carbinolamine that led to the accumulation of a carbonyl product during the reaction.

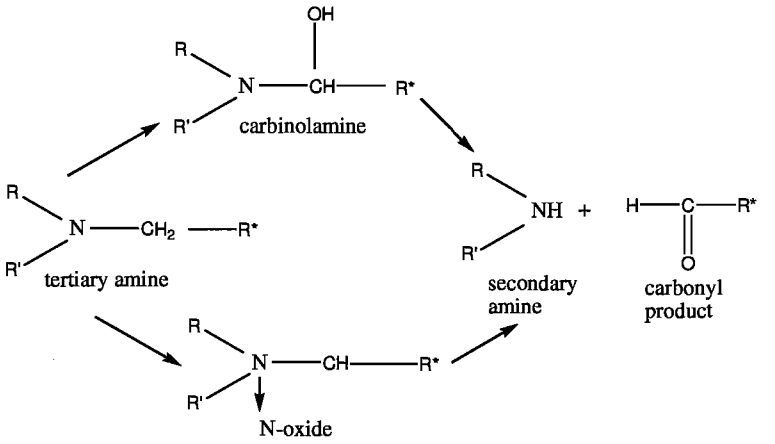


Figure 25: Mechanisms[98] for *N*- dealkylation of tertiary amines

Since the carbonyl product could inhibit the enzyme reaction, semicarbazide-HCl was added to enzyme assays to trap this inhibitory intermediate. The development of a successful assay required an extraction procedure similar to our protocols except that cells were ground with a pinch of sea sand using a mortar and pestle and the buffer

used for extraction was 100 mM Tris-HCl (pH 7.5) with 14 mM β-mercaptoethanol. This extraction method with *Cunninghamella echinulata* cells was repeated and enzyme assays were repeated in the presence of semicarbazide-HCl at 2 separate concentrations (Appendix 1, Table 4). Unfortunately, none of the assays produced any reaction products as already discovered in the studies described in Tables 1 to 3. If a reaction product was being produced, the methods used for its detection did not appear to be sensitive enough to identify it.

Since the detection limit for northebaine of the TLC assay has been reported to be 1000 ng[106], Ultra Performance Liquid Chromatography- Mass Spectrometry (UPLC-MS) which can detect as little as 16 ng was used as a more sensitive tool for product identification (Figure 24). Another key feature of UPLC- MS was that the chromatograph only requires 10 minutes to identify the reaction product by its retention time, its absorbance spectrum and its molecular mass, respectively. Unfortunately this method did not identify a clear northebaine reaction product in the enzyme assays described in Appendix 1, in Tables 1-5.

In order to develop an even more sensitive assay than those using TLC or UPLC-MS (Figure 24), the radioactive substrate, 3[$^{14}CH_3$]-thebaine (Specific activity, 2.035 Gbq/mmol or 55 mCi/mmol) was obtained.

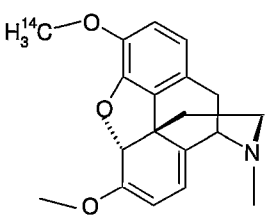


Figure 26: Structure of 3[$^{14}CH_3$]-thebaine used for the present study

With this substrate, the sensitivity of the assay could be improved by at least one order of magnitude over the UPLC-MS assay.

3.3.5 Biotransformation of 3[$^{14}CH_3$]-thebaine into 3[$^{14}CH_3$]-northebaine by cultures of *Cunninghamella echinulata*

Previous enzymatic studies with cell-free extracts of *Cunninghamella bainieri* suggested that *N*-demethylation of codeine occurs through the membrane-associated cytochrome-P450-dependent monooxygenase. Although cultures of *C. echinulata* were extracted by means of identical protocols, none of the conditions used by Gibson[98] produced northebaine as a reaction product when analyzed by UPLC or by thin-layer chromatography (TLC) (Figure 24, Appendix Tables 1 to 5). In order to develop a radioactive enzyme assay using, 3[$^{14}CH_3$]-thebaine as a substrate, this substrate was first tested in biotransformation studies (Figure 26). As described previously (Figures 19-22), a lag phase of 2 days was also observed in the biotransformation of 3[$^{14}CH_3$]-thebaine into 3[$^{14}CH_3$]-northebaine. This is clearly seen in the TLC autoradiogram where 3[$^{14}CH_3$]-northebaine begins to appear by day 3 after substrate addition and in the % conversion of 3[$^{14}CH_3$]-thebaine into 3[$^{14}CH_3$]-northebaine that was observed when each radioactive spot was counted (Figure 27). The results show that 3[$^{14}CH_3$]-thebaine was converted with time essentially quantitatively to 3[$^{14}CH_3$]-northebaine with similar kinetics to those observed in non-radioactive biotransformation studies (Figures 19 to 22). An alternative mechanism postulated by Abel[99] suggested that *N*-demethylation may proceed by transfer of the *N*-methyl group to the phenolic OH group on C-3 by a regiospecific *N*- to *O* methyltransferase. In biotransformation studies with 3[$^{14}CH_3$]-thebaine, the

mechanism proposed by Abel[99] should have involved loss of the label for regiospecific *N*- to *O*-methyltransferase activity to occur. Since 3[$^{14}CH_3$]-thebaine was almost quantitatively recovered during this biotransformation to 3[$^{14}CH_3$]-northebaine, it seems unlikely that *N*-demethylation would occur by this mechanism.

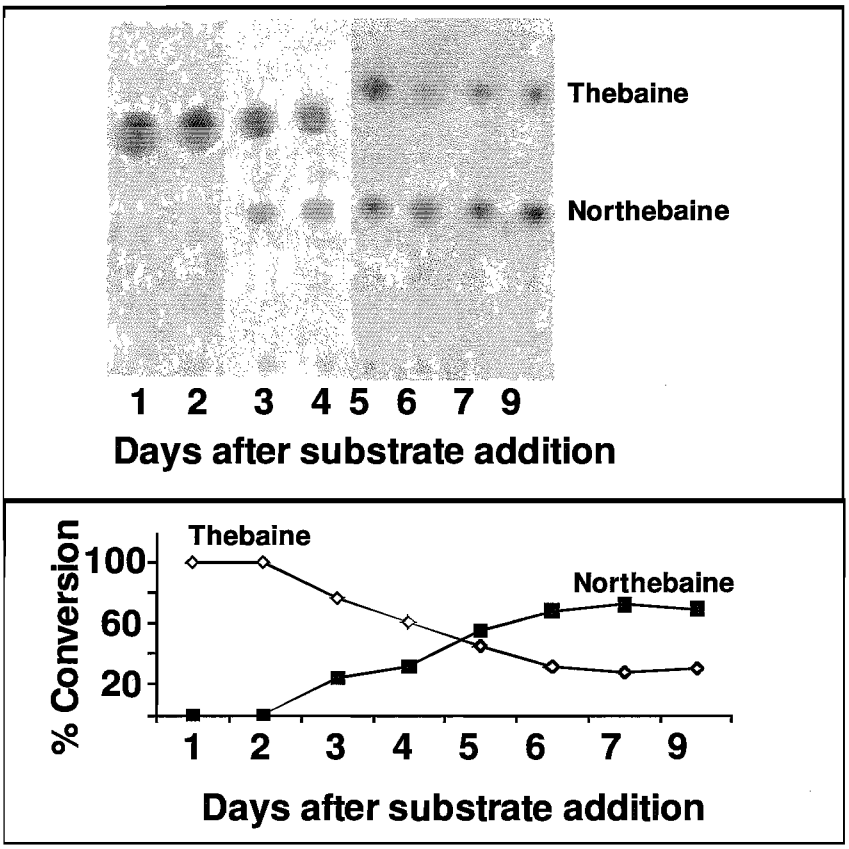


Figure 27: Conversion of [$^{14}CH_3$] thebaine into 3[$^{14}CH_3$] northebaine by *C. echinulata.*

In order to better reproduce the results of Gibson[98], a novel highly sensitive assay using 3[$^{14}CH_3$]-thebaine was also developed for detection of labeled northebaine in cell-free extracts of *C. echinulata.* While in biotransformation studies (Figure 27), cell-free extracts assayed as described by Gibson, were

totally ineffective in producing this compound. Nevertheless, the availability of 3[$^{14}CH_3$]-thebaine will be extremely important to eventually develop an assay for this reaction and to verify what type of biochemical reaction is responsible for this biotransformation in *C. echinulata.*

3.3.6 Cell free extracts assayed under different conditions do not convert 3[$^{14}CH_3$]-thebaine into 3[$^{14}CH_3$]-northebaine

The biotransformation studies (Figure 26) showed that *C. echinulata* efficiently converted 3[$^{14}CH_3$]-thebaine into 3[$^{14}CH_3$]-northebaine. In order to better reproduce the results of Gibson, a novel highly sensitive assay using 3[$^{14}CH_3$]-thebaine was developed for detection of labeled northebaine in cell-free extracts of *C. echinulata.* However, cell-free extracts assayed as described by Gibson, were totally ineffective in producing this compound. Nevertheless, the availability of 3[$^{14}CH_3$]-thebaine will be extremely important to eventually develop an assay for this reaction and to verify what type of biochemical reaction is responsible for this biotransformation in *C. echinulata.*

3.3.6.1 Development of an extraction protocol to remove fungal contaminants interfering with TLC separation of radiolabeled alkaloid.

The Rf of standard 3[$^{14}CH_3$]-thebaine was 0.5 as determined by TLC and autoradiography (Section 3.3.8.5 in Materials and Methods) (Figure 28A, lane 1 and 27B, lane 1). Initial enzyme assays (Section 3.3.8.7, Method 2 & 3 in Materials and Methods) with cell free extracts involved stopping the reaction with 1N HCl to bring

the pH down to 2 and by extracting the hydrophobic fungal contaminant in ethyl acetate 3 times. Under these conditions the labeled alkaloids were not extracted. After removal of each ethyl acetate layer, the aqueous layer was treated with 2M NaOH to bring the pH up to 10 and re-extracted with ethyl acetate 3 times. This procedure seemed to extract additional compounds of fungal origin that interfered with the TLC (Figure 28A lanes 2 to 7) whereas incubation of 3[$^{14}CH_3$]-thebaine in the presence of various substrates but without fungal extract produced the expected radioactive spot with an identical Rf to that of 3[$^{14}CH_3$]-thebaine (Figure 28A, compare lanes 1 and 8) without production of further non-enzymatic reaction products.

The retardation caused by fungal contaminants was resolved by stopping enzyme assays with 2M NaOH and extracting 3 times with ethyl acetate at pH-10. After concentrating organic layer down to 0.5 mL, an equal amount of water was added and the sample was acidified to pH-2-3 using 1N HCl and extracted three times in ethyl acetate. The aqueous layer was basified as described previously, extracted 3 times in ethyl acetate and dried *in vacuo* for TLC after dissolving the residue in 10 μL of methanol. Under these conditions, the TLC resolved the expected radioactive spot with an identical Rf to that of 3[$^{14}CH_3$]-thebaine, but no clear 3[$^{14}CH_3$]-northebaine reaction products with an Rf of 0.25 could be identified under the reaction conditions utilized (Figure 28B, lanes 2 to 8).

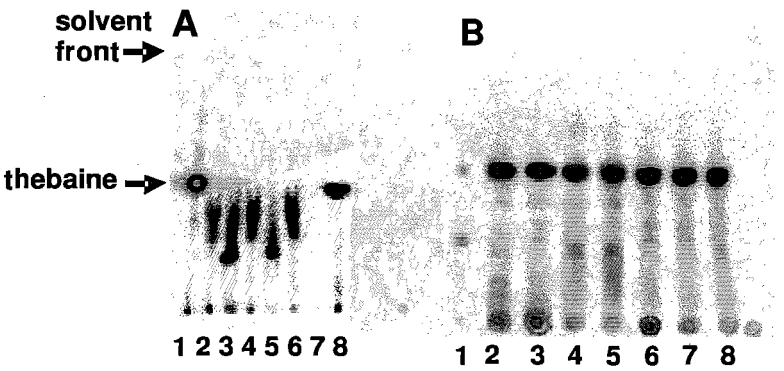


Figure 28: Development of a TLC autoradiography system for separation and analysis of 3[$^{14}CH_3$]-thebaine and 3[$^{14}CH_3$]-northebaine. A. Retardation of chromatographic behaviour of radioactive alkaloids by a fungal contaminant extracted by ethyl acetate treatment. B. Removal of the fungal contaminant with an alternative extraction procedure. A, Lane 1 is 3[$^{14}CH_3$]-thebaine standard (0.1 μCi); Lane 2 to 6, enzyme assays with 3[$^{14}CH_3$]-thebaine, lane 7 is enzyme assay without 3[$^{14}CH_3$]-thebaine, Lane 8 is assay without cell free extract. B. Lane 1. 3[$^{14}CH_3$]-thebaine and 3[$^{14}CH_3$]-northebaine obtained from whole cell *C. echinulata* biotransformation of 3[$^{14}CH_3$]-thebaine. Lanes 2 to 8 enzyme assays by elimination of one co-factor at a time.

3.3.7 Various extraction procedures for proteins and co-factor combinations tested in the present study

While none of the assay conditions (Figure 28) produced a reaction product, this extraction protocol and TLC method was used extensively, to repeat the assays with 3[$^{14}CH_3$]-thebaine that had already been performed with non-radioactive thebaine as a substrate. Unfortunately, none of these experiments were successful to identify the conditions required for cell free biosynthesis of northebaine from thebaine.

3.3.8 Materials and Methods for enzyme assays using 3[$^{14}CH_3$] thebaine as substrate

3.3.8.1 Organism

Cunninghamella echinulata (ATCC 9244) was purchased from American Type Culture Collection (ATCC) USA.

3.3.8.2 Test Compounds

Thebaine was provided by Noramco Inc. USA. The 3[$^{14}CH_3$]-thebaine (Specific activity 2.035 Gbq/mmol; 99% purity) was purchased from American Radiolabeled Chemicals, USA. The purity of 3[$^{14}CH_3$]-thebaine was checked by co-chromatography of the radioactive compound with pure thebaine using TLC in 2 separate solvents (Toluene: Ethyl acetate: Diethylamine in the ratio 70: 20: 10 and Dichloromethane: MeOH: NH_4OH in the ratio 92: 8: 1. Autoradiography of these TLCs revealed that the sample contained a single radioactive spot corresponding to pure thebaine.

3.3.8.3 Chemicals and reagents

Sorensen phosphate buffer was prepared from Analar grade KH_2PO_4 and Na_2HPO_4,

which was purchased from Sigma- Aldrich Chemical Co. Oakville, Canada. Triton X-100, semicarbazide- HCl , NADH, NADPH and $FeSO_4$. $7H_2O$ was also obtained from Sigma-Aldrich.

3.3.8.4 Culture media

Cunninghamella echinulata was maintained on potato dextrose agar slants at 4 °C. Biotransformation broth media contained 2% glucose, 5% corn steep liquor and 1% nutrient broth (GCN broth) at pH-5.0 that was autoclaved at 121 °C for 15 minutes. 1 mL spore suspension (prepared by adding 5 mL sterile distilled water into one agar slant of culture) was used to inoculate a baffled flask containing 200 mL biotransformation broth media. The cultures were incubated at 26 °C on a rotary shaker (160 rev/ min) for 2 days to produce the pelleted growth form (Figure 10). Thebaine dissolved in a minimal amount of 1N HCl was added (0.2 mg thebaine /mL) to the cultures to induce demethylase activity as suggested by previous experiments (Figures 19-22). Fungal cell cultures cultivated for were ~85 hours before harvesting them by filtration and washing them 3 times with Sorensen's phosphate buffer (KH_2PO_4 and Na_2HPO_4, 0.66 M, pH- 7.0 with 14 mM β-mercaptoethanol) to give approximately 10g fungal pellets. These pellets were either used directly to produce cell-free extracts for enzyme assay or they were frozen in liquid nitrogen and stored in a -80 °C freezer.

3.3.8.5 Purity of 3[$^{14}CH_3$]-thebaine

The 3[$^{14}CH_3$]-thebaine (Specific activity 2.035 Gbq/mmol)was produced by American Radiolabeled Chemicals. The purity of 3[$^{14}CH_3$]-thebaine was checked by co-chromatography of the radioactive compound with pure thebaine using TLC in 2 separate solvents (Toluene: Ethyl acetate: Diethylamine in the ratio 70: 20: 10 and

Dichloromethane: MeOH: NH_4OH (92:8:1). Autoradiography of these TLCs revealed that that the sample contained a single radioactive spot corresponding to pure thebaine.

3.3.8.6 Time profile of biotransformations with 3[$^{14}CH_3$]-thebaine

C. echinulata was grown in 25 mL GCN broth for 48 hours at 26 °C and 125 rpm to produce pellet form of fungal cells in baffled flasks (Figure 10). At this point the culture was inoculated 0.5 μCurie 3[$^{14}CH_3$]-thebaine together with 12.5 mg unlabelled thebaine. Samples (1 mL) were harvested for analysis every 24 hr for biotransformation product formation. Samples were titrated with NaOH to pH~10, extracted for alkaloids into EtOAc and the EtOAc was evaporated to dryness. Samples were dissolved in 10 ml methanol and were submitted to TLC. The spots corresponding to 3[$^{14}CH_3$]-thebaine (Rf 0.5) and 3[$^{14}CH_3$]-northebaine (Rf 0.25) were harvested from the TLC plates and quantified by counting in a liquid scintillation counter.

3.3.8.7 Preparation of cell free extracts

Method 1

Cells free extracts for the initial experiments were prepared from fungal pellets stored at -80 °C. The frozen cells were ground using mortar and pestle in liquid nitrogen and the total proteins extracted using 100mM Tris-HCl buffer, pH 7.5 containing 14mM β-mercaptoethanol as reducing agent. The extraction was done at 4 °C. The crude extract was filtered through a layer of mira cloth and was centrifuged at 500g for 10 minutes at 4 °C. The supernatant was desalted through PD10 column and the filtrate was used for enzyme assays. The incubation of enzyme was done at 37 °C with the substrate and co-factors for one hour.

Method 2

The fungal pellets from freshly harvested cells were ground at 4 °C to a paste (using mortar and pestle with a pinch of sand) in 1mM DTT (dithiothreitol), 14 mM β-mercaptoethanol, 15% glycerol and 50mM Tris HCl at pH-7.5. The cells were sonicated (using a sonicator provided by Fisher Scientific Co. USA) at speed 4 continuous, for one minute X 4 with intermittent cooling on ice for one minute. After sonication, the crude extract was filtered through a layer of mira cloth and was centrifuged at 500g for 10 minutes at 4 °C. The supernatant was desalted through PD10 column and the filtrate was used for enzyme assays. The incubation of enzyme was done at 37 °C with the substrate and co-factors for one hour.

Method 3

The fungal pellets from freshly harvested cells were ground at 4 °C to a paste (using mortar and pestle with a pinch of sand) with 10 mL phosphate buffer and the homogenate was transferred to a 50 ml centrifuge tube. The cells were sonicated (using a sonicator provided by Fisher Scientific Co. USA) at speed 4 continuous, for one minute X 4 with intermittent cooling on ice for one minute. The homogenate was filtered through a layer of mira cloth and washed with 10 mL phosphate buffer X 2 into a precooled 125 mL Erlenmeyer flask to which Triton X-100 solution (10% w/v) was added to give a surfactant concentration of 0.33% w/v. The mixture was agitated on a radial shaker at 4 °C for 30 minutes and the suspension was immediately centrifuged at 32,000 g for 20 minutes at 4 °C to remove the cell debris. The supernatant was decanted to give the cell free extract which was used for enzyme assays.

3.3.8.8 Estimation of total proteins in cell free extracts

Total protein concentration in the cell free extracts was determined by the Bradford's method Reagents: Bradford's reagent- 40 µL, Protein solution- 10 µL, Millipore water- 150 µL, Reaction time – 15 to 30 minutes. The reaction was measured using spectrophotometer at 595nm.

3.3.8.9 Estimation of *N*-demethylase activity

A typical example of a set of enzyme assays performed in the present study using radio-labeled thebaine is given in the table below (Table 2). The concentration of total proteins present in the fungal extract was 2mg/mL.

Table 2: Enzyme assays by elimination of one co-factor at a time.

Sample	1	2	3	4	5	6	7	8
Protein (2mL)	+	+	+	+	+	+	+B	+
14C Thebaine (0.1 µCi)	+	+	+	+	+	+	+	-
Thebaine (100 µg)	+	+	+	+	+	+	+	-
NADPH (1mM)	+	+	+	+	-	-	+	-
NADH (1mM)	+	+	+	-	+	-	+	-
Ferrous (5mM)	+	+	-	+	+	-	+	-
Semicarbazide-HCl (15mM)	+	-	+	+	+	-	+	-

3.3.8.10 Thin Layer Chromatography (TLC) determination of 3[$^{14}CH_3$]-northebaine

TLC solvent: Toluene: Ethyl acetate: Diethyl amine in the ratio 70: 20: 10. After the enzyme assays the reaction was stopped by adding 2N NaOH to get pH~10. The samples were processed by extracting the alkaloids into EtOAc (4 mL X 4) at pH-10. The combined organic layers were dried *in vacuo* & re-dissolved in 1mL EtOAc. 500 µL milli Q H_2O was added to the EtOAc extract, acidified using acetic acid and extracted twice. The aqueous layer was basified and extracted again using EtOAc (4mL x 3).The last basic organic extracts were dried *in vacuo* & used for TLC. The TLC was exposed to phosphor screen (purchased from Amersham Scientific Co., USA) overnight and the images were developed by a Fujifilm image reader. Quantification of the 3[$^{14}CH_3$]-northebaine formed if any was done by scraping the corresponding TLC spot and subjecting it to scintillation counting using Beckman's Scintillation counter.

4. Conclusions and future work

In the course of the studies, we discovered the capability of *Helicostylum piriforme*, to transform thebaine to *nor*-thebaine. We established the versatility of *Cunninghamella echinulata* to perform *N*-demethylation of a variety of morphine alkaloids. Even though the genus *Cunninghamella* is speculated to biotransform morphine alkaloids[81], none of the morphinans other than codeine and a thebaine derivative (buprenorphine) had been tested so far (Table 1). Our results indicate that *Cunninghamella echinulata* is truly a microbial model for mammalian drug metabolism. It can also be concluded that when there is a ketone at the C-6 position, fungal strains tend to reduce the ketone in varying yields depending on the alkaloid and the particular fungus being used. The only exception to this occurred with *Cunninghamella* that *N*-demethylated both hydrocodone and oxycodone which are both ketone at the C-6 position. It is worth noting that the morphology of the fungi also has an influence on the yield of biotransformation (Table 1). When *Cunninghamella echinulata* grew as single large biomass, the biotransformation yield of *nor*-thebaine was approximately three times lower when compared to that obtained when it grew as small pellets (picture: p28). The small pellet morphology increases the surface area of contact with the broth containing the substrate for biotransformation. This increases the chances of absorption of the substrate and the incorporation of oxygen into cells. It is assumed that these factors contribute to the increase in yield.

A possible industrial application of fungal *N*-demethylation might involve immobilization of the enzyme(s), to allow a continuous yield of the *N*-demethylated product. But this method may require the addition of expensive co-factors to the

reaction media. An alternative to this method is to identify the gene and clone it to produce a recombinant *E.coli* expressing this gene. The recombinant *E.coli* can be grown in large fermenters and the bacterial cell will provide the co-factors necessary to perform the reaction, thus saving the addition of co-factors to the media.

It has been demonstrated in the past that both viable and cell-free extracts of *Cunninghamella bainieri* are capable of *N*-demethylating codeine.[71] It has also been reported that the *N*-demethylase is probably membrane bound cytochrome P450 monooxygenase. However, all our attempts to repeat the *N*-demethylation of thebaine using cell free extracts of *Cunninghamella echinulata* were unsuccessful. We were unable to reproduce the *N*-demethylation reaction performed by the cell extracts of *C. bainieri* even after having a powerful tool like radioactive substrate, when [^{14}C] thebaine was used as substrate.

Future work in the area of engineering a recombinant *E.coli* can proceed in two ways either using the gene from fungus or using the gene from mammalian cell lines. If an enzyme assay could be developed for testing the conversion of thebaine to *nor*-thebaine using cell free extracts of *Cunninghamella echinulata*, then we can characterize the enzyme and clone the gene. If it is a soluble enzyme, then isolation of the protein will be easier compared to a membrane- bound cytochrome P450 monoxygenase as reported earlier. If the enzyme is membrane bound, then probably isolation of the gene transcripts using degenerate primers of known cytochrome P450 demethylases will be the focus. Designing the primers, cloning and characterization of each of the transcript can be a very time-consuming effort. It is known that human liver microsomes are capable of *N*-demethylating morphine alkaloids. The human

cytochrome P450 demethylases can be cloned and tested for *N*- demethylation of morphine alkaloids. This second approach may provide results faster than isolating enzyme from the fungus.

5. Experimental Section

5.1 General Experimental Details

Liquid reagents were distilled prior to use, and commercial solids were used as supplied. Analytical thin-layer chromatography was performed on Silicycle 60 Å 250 μm TLC plates with F-254 indicator. Flash column chromatography was performed using Natland 200-400 mesh silica gel. Melting points were recorded on a Hoover Unimelt apparatus and are uncorrected. IR spectra were obtained on a Perkin_Elmer One FT-IR spectrometer. 1H- and 13C-NMR spectra were recorded on a Bruker (300 MHz or 600 MHz) spectrometer. All chemical shifts are referenced to TMS or residual undeuterated solvent (CHCl3, DCM). Separation by HPLC was either performed on a Hitachi L-6000 HPLC using a Hitachi L-4000H UV detector (254 nm) with a Phenomenex primespher 5 C18 HC 250 x 10 mm column; 2 ml/min flow; 5 mM KH2PO4, 0.1% NEt3, pH = 2.8 adjusted with 2N HCl : MeOH (80:20) or performed on an Agilent 1100 series HPLC using a Phenomenex primesphere 5 C18 HC, 150 x 4.6 mm column and 1.3 ml/min flow. Compounds were detected at 280 nm and opioids were eluted with a gradient of 5 mM KH2PO4, 0.1% NEt3, pH = 2.8 adjusted with 2N HCl and MeOH. Mass spectra were recorded on Kratos/MsI Concept 1S mass spectrometer at Brock University.

5.2 Experimental procedures

Maintenance of fungi and biotransformation protocols

5.2.1.0. ***Cunninghamella echinulata*****, ATCC 9244** was propagated on **potato dextrose agar** (ATCC medium 336) slants at **26°C**. Mature slants (>7 days) were used for the preparation of spore suspensions.

5.2.1.1. Fermentation/Biotransformation media

Batch fermentations were carried out at **26°C** with vigorous aeration (**150 rpm** in baffled flasks on an orbital shaker) for up to **15 days. GCN medium** (glucose, 2%; corn steep liquor, 5%; Difco nutrient broth, 1%; pH 5.0) was used to promote high levels of *N*-demethylation.[91]

5.2.1.2. Biotransformation protocols

Typically, biotransformations were carried out in 200 ml fermentation media in 1L reaction vessels. All media were seeded with 0.5ml concentrated spore suspension made up in sterile distilled water (typically 3ml for 1 slant). Substrate, thebaine, hydrocodone, codeine, morphine, oxycodone or oripavine, (100 mg ml^{-1} dissolved in 1N HCl except in the case of codeine. Codeine phosphate, 150mg ml^{-1} was dissolved in distilled water) was added to the fermentation medium to give a final concentration of 0.5mg ml^{-1} 24 to 48 hours post-inoculation. Progress of the biotransformation was followed by TLC as described below.

5.2.1.3. TLC and sample preparation

The progress of the alkaloid demethylation reaction was monitored by TLC in a solvent system comprising of DCM: MeOH: NH_4OH (92: 8: 1, v/v/v). Samples for analysis were prepared by mini work-up. A mini work-up was performed as follows:

2ml of broth was centrifuged, the supernatant was basified using conc.NH_4OH and then extracted with dichloromethane (DCM).

5.2.1.4. Analysis of products

After a biotransformation was complete, the broth was separated from fungi by centrifugation, followed by filtration. The broth was basified to pH 10 using conc. NH_4OH (except in the case of morphine for which pH was adjusted to 8) and extracted with chloroform (3×250ml). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated *in vacuo*. The biotransformation products were separated by flash column chromatography on silica gel using the eluents $CHCl_3$ and MeOH. The products were identified by NMR (^{13}C & ^{1}H), IR, MS and HPLC, and matched to literature data when possible.

5.2.2.0. ***Trametes sanguinea*****, ATCC 14622**, was propagated on **yeast mold agar** (ATCC medium 200) slants at **26°C**. Mature slants (>7 days) were used for the preparation of spore suspensions.

5.2.2.1. Fermentation media

Batch fermentations were carried out at **30°C** with vigorous aeration (**160 rpm** on an orbital shaker) for up to **22 days. M-2 medium**[92] (glucose, 1%; peptone, 0.2%; beef/meat extract, 0.1%; yeast extract, 0.1%; corn steep liquor, 0.3%; pH 5.0)[7] was used for biotransformation.[67-69]

5.2.2.2. Biotransformation protocols

Typically, biotransformations were carried out in 200 ml fermentation media in 1 L reaction vessels. All media were seeded with 0.5ml concentrated spore suspension made in sterile distilled water (typically 3ml for 1 slant). Substrate (100mg ml^{-1}

dissolved in 1N HCl except in the case of codeine. Codeine phosphate, 150mg ml^{-1} was dissolved in distilled water) was added to the fermentation medium to a final concentration of 0.5mg ml^{-1}, 4 days post-inoculation. Progress of the biotransformation was followed by TLC.

TLC, sample preparation and analysis of products are same as in the case of *Cunninghamella echinulata.*

5.2.3.0. ***Trametes cinnabarina*****, ATCC 14623,** was propagated in **yeast mold broth** (ATCC medium 200) at **26°C**. Mature cultures (>7 days growth) were used for the inoculation of biotransformation media. The culture was maintained on yeast mold agar slants.

5.2.3.1. Fermentation media

Batch fermentations were carried out at **30°C** with vigorous aeration (**160 rpm** on an orbital shaker) for up to **9 days**. **NRM or nutrient rich medium** (Glucose, 0.5%; KH_2PO_4, 0.05%; L-asparagine, 0.052%; Yeast Extract, 0.05g; KCl, 0.05g; $MgSO_4$ x $7H_2O$, 0.05%; $FeSO_4$, 0.001% ; dissolved in 950mL distilled water and 50mL mineral salt solution was added. pH- 4.5; mineral salt solution- $Mn(CH_3COO)_2$ x $4H_2O$, 8mg; $CuSO_4$ x $5H_2O$, 3mg; $ZnSO_4$ x $7H_2O$, 2mg; $Ca(NO_3)_2$ x $4H_2O$, 50mg; d/w, 50mL) was used for biotransformation.[70]

5.2.3.2. Biotransformation protocols

Typically, biotransformations were carried out in 200 ml fermentation media in 1L reaction vessels. All media were seeded with 1mL mature culture. Cultures were static during the growth period. Substrate (100mg ml^{-1} dissolved in 1N HCl except in the case of codeine. Codeine phosphate, 150mg ml^{-1} was dissolved in distilled water) was

added to the fermentation medium to a final concentration of 0.5mg ml^{-1}, 7 days post-inoculation. Progress of the biotransformation was followed by TLC. TLC, sample preparation and analysis of products are same as in the case of *Cunninghamella echinulata.*

5.2.4.0. ***Helicostylum piriforme*****, ATCC 8992** was propagated on **cornmeal yeast glucose agar** ,CMYG (ATCC medium 310) slants at **24°C.** Mature slants (>7 days) were used for the preparation of spore suspensions.

5.2.4.1. Fermentation/Biotransformation media

Batch fermentations were carried out at **26°C** with vigorous aeration (**125 rpm** in baffled flasks on an orbital shaker) for up to **15 days. Glucose Corn steep liquor Salt, GCS medium** (Glucose, 3%; Corn Steep Liquor, 1%; K_2HPO_4, 0.2%; KH_2PO_4, 0.1%; $NaNO_3$, 0.2%; KCl, 0.05%, $MgSO_4$ * 7 H_2O, 0.05%, $FeSO_4$ * 7 H_2O, 0.002%), was used for biotransformation.[83]

5.2.4.2. Biotransformation protocols

Typically, biotransformations were carried out in 200 ml fermentation media in 1L reaction vessels. All media were seeded with 0.5ml concentrated spore suspension made up in sterile distilled water (typically 3ml for 1 slant). Substrate (100mg ml^{-1} dissolved in 1N HCl except in the case of codeine. Codeine phosphate, 150mg ml^{-1} was dissolved in distilled water) was added to the fermentation medium to give a final concentration of 0.5mg ml^{-1} 24hours post-inoculation. Progress of the biotransformation was followed by TLC. TLC, sample preparation and analysis of products are same as in the case of *Cunninghamella echinulata*

5.2.5.0. ***Curvularia lunata*****, ATCC 12017** was propagated on **Rabbit food agar** (ATCC medium 340) slants at **24°C**. Mature slants (>14 days) were used for the preparation of spore suspensions.

5.2.5.1. Fermentation/Biotransformation media

Batch fermentations were carried out at **27°C** with vigorous aeration (**150 rpm** in baffled flasks on an orbital shaker) for up to **10 days. Beef Extract Medium, BEM** (Glucose, 0.5%; Peptone, 0.1%; Yeast Extract, 0.1%; Beef Extract, 0.1%) was used for biotransformation.[93]

5.2.5.2. Biotransformation protocols

Typically, biotransformations were carried out in 200 ml fermentation media in 1L reaction vessels. All media were seeded with 0.5ml concentrated spore suspension made up in sterile distilled water (typically 3ml for 1 slant). Substrate (100mg ml^{-1} dissolved in 1N HCl except in the case of codeine. Codeine phosphate, 150mg ml^{-1} was dissolved in distilled water) was added to the fermentation medium to give a final concentration of 0.5mg ml^{-1} 48hours post-inoculation. Progress of the biotransformation was followed by TLC. TLC, sample preparation and analysis of products are same as in the case of *Cunninghamella echinulata*

5.2.6.0 ***Sporotrichum sulfurescens/ Beauveria bassiana*****, ATCC 7159** was propagated on **Potato Dextrose Agar, PDA** (ATCC medium 336) slants at **24°C**. Mature slants (>7 days) were used for the preparation of spore suspensions.

5.2.6.1. Fermentation/Biotransformation media

Batch fermentations were carried out at **28°C** with vigorous aeration (**150 rpm** in baffled flasks on an orbital shaker) for up to **7 days. Glucose, Corn steep liquor**

medium (Glucose, 1%; Corn steep liquor, 2%, and tap water not distilled water) was used for biotransformation.[86]

5.2.6.2. Biotransformation protocols

Typically, biotransformations were carried out in 200 ml fermentation media in 1L reaction vessels. All media were seeded with 0.5ml concentrated spore suspension made up in sterile distilled water (typically 3ml for 1 slant). Substrate (100mg ml^{-1} dissolved in 1N HCl except in the case of codeine. Codeine phosphate, 150mg ml^{-1} was dissolved in distilled water) was added to the fermentation medium to give a final concentration of 0.5mg ml^{-1} 4 days post-inoculation. Progress of the biotransformation was followed by TLC. TLC, sample preparation and analysis of products are same as in the case of *Cunninghamella echinulata*

5.3. General experimental details for enzyme assays

5.3.1. Bradford's Assay for determining the concentration of total proteins

Reagents:Bradford's reagent - 40μL, Protein solution -10μL,

Millipore water -150 μL, Reaction time – 15 to 30 minutes.

The reaction was measured using spectrophotometer at 595nm

5.3.2. Thin layer chromatography (TLC) for enzyme assays

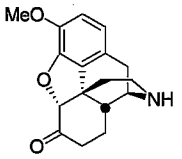
TLC solvent: Toluene: Ethyl acetate: Diethyl amine in the ratio 70: 20: 10

Staining was done using Marquis reagent by spray technique

5.3.3. Marquis reagent: Sulfuric acid – 100mL, Formaldehyde – 3mL

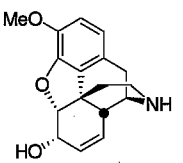
R_f values : Thebaine – 0.58, *Nor*thebaine – 0.26, Oripavine - 0.30, *Noro*ripavine – 0.18.

5. 4. Biotransformation product identification



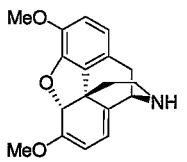
***Nor*hydrocodone (34).**

The title compound **34** was isolated following the protocol for biotransformations using *Cunninghamella echinulata*, ATCC 9244 in 10% yield as colorless solid. Data for **34** are identical to those published in the literature.[94] mp 149 - 151 °C (MeOH/diethyl ether); R_f 0.25 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -83. 988° (c = 0.215, $CHCl_3$), $[\alpha]_D20$ = -54.823° (c = 0.58, MeOH); literature value is $[\alpha]_D11$ = -4.0° (c = 1.0, MeOH)[100]; IR (film) ν_{max}: 3369, 2928, 1725, 1636, 1609, 1504, 1439, 1274, 1061 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ: 6.74 (d, *J* = 8.2 Hz, 1H), 6.67 (d, *J* = 8.2 Hz, 1H), 4.66 (s, 1H), 3.93 (s, 3H), 3.48 - 3.52 (m, 1H), 2.85 - 2.95 (m, 2H), 2.79 (d, *J* = 18.5 Hz, 1H), 2.71 - 2.77 (m, 1H), 2.55 (dt, *J* = 12.6, 3.2 Hz, 1H), 2.45 (dt, *J* = 13.8, 4.6 Hz, 1H), 2.40 (td, *J* = 13.8, 4.6 Hz, 1H), 1.97 (td, *J* = 12.3, 4.8 Hz, 1H), 1.82 - 1.91 (m, 2H), 1.22 (qd, *J* = 13.3, 3.2 Hz, 1H); ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 207.6, 145.5, 142.9, 127.3, 126.3, 119.9, 114.7, 91.6, 56.8, 52.4, 47.7, 43.0, 40.3, 39.0, 36.0, 30.8, 25.8 ppm; MS (EI) *m/z* (%) 285 (9), 87 (11), 86 (21), 85 (65), 84 (35), 83 (100), 49 (14), 48 (13), 47 (33); HRMS (EI) calcd for $C_{17}H_{19}NO_3$: 285.1365, found 285.1364.



***Nor*codeine (33).**

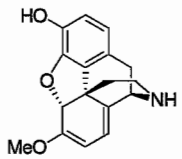
The title compound **33** was isolated following the protocol for biotransformations using *Cunninghamella echinulata*, ATCC 9244 in 10% yield as colorless solid. Data for **33** are identical to those published in the literature.[95] mp 182 - 183 °C (MeOH/diethyl ether); R_f 0.22 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -90.971° (c = 0.224, $CHCl_3$); literature value is $[\alpha]_D28$ = +115.23° (c = 1.09, $CHCl_3$)[101](enantiomer); IR (film) ν_{max}: 3309, 3000, 2935, 2837, 1635, 1603, 1504, 1453, 1282, 1127, 943 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ: 6.70 (d, *J* = 8.2 Hz, 1H), 6.60 (d, *J* = 8.2 Hz, 1H), 5.72 - 5.76 (m, 1H), 5.28 (dt, *J* = 9.9, 2.5 Hz, 1H), 4.89 (d, *J* = 6.4 Hz, 1H), 4.17 - 4.22 (m, 1H), 3.87 (s, 3H), 3.65 - 3.69 (m, 2H), 2.98 (td, *J* = 12.1, 4.4 Hz, 1H), 2.86 - 2.93 (m, 2H), 2.83 (d, *J* = 18.7 Hz, 1H), 2.59 - 2.63 (m, 1H), 1.88 - 1.98 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 146.4, 142.2, 133.7, 131.1, 128.2, 127.4, 119.6, 112.8, 91.9, 66.3, 56.3, 52.0, 43.9, 41.3, 38.5, 36.6, 31.4 ppm; MS (EI) *m/z* (%) 285 (39), 87 (13), 86 (17), 85 (70), 84 (25), 83 (100), 82 (10), 59 (34), 49 (11), 47 (25), 45 (13), 44 (29), 43 (19), 42 (12); HRMS (EI) calcd for $C_{17}H_{19}NO_3$: 285.1365, found 285.1368.



***Nor*thebaine (37).**

The title compound **37** was isolated following the protocol for biotransformations using *Cunninghamella echinulata*, ATCC 9244 and *Helicostylum piriforme,* ATCC 8992, in 35-50% and 39% yield respectively, as colorless solid. Data for **37** are

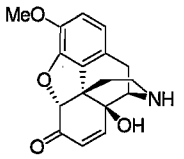
identical to those published in the literature.[95] mp 109 -111 °C (MeOH/diethyl ether); R_f 0.59 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -197.956° (c = 0.31, 5% MeOH in $CHCl_3$); literarture value is $[\alpha]_D23$ = -200° (c = 0.1, 5% MeOH in $CHCl_3$)[82]; IR (film) ν_{max}: 3295, 3004, 2931, 2837, 1668, 1607, 1505, 1447, 1234, 1019 cm^{-1}; ^1H NMR (MeOH-d_4, 600 MHz) δ: 6.74 (d, *J* = 8.2 Hz, 1H), 6.64 (d, *J* = 8.2 Hz, 1H), 5.63 (d, *J* = 6.5 Hz, 1H), 5.27 (s, 1H), 5.15 (d, *J* = 6.5 Hz, 1H), 4.00 (d, *J* = 6.4 Hz, 1H), 3.84 (s, 3H), 3.62 (s, 3H), 3.07 - 3.19 (m, 3H), 2.94 (dd, *J* = 13.4, 4.5 Hz , 1H), 2.14 (dt, *J* = 12.9, 5.0 Hz, 1H), 1.79 (dd, *J* = 13.2, 2.8 Hz 1H) ppm; ^{13}C NMR (MeOH-d_4 150 MHz) δ: 152.9, 144.8, 142.8, 133.2, 131.6, 127.4, 119.2, 113.7, 111.7, 95.5, 88.7, 55.8, 54.0, 53.4, 46.1, 38.4, 37.5, 36.2, ppm; MS (EI) *m/z* (%) 298 (3), 297 (13), 88 (19), 86 (65), 84 (100), 49 (16), 47 (21), 45 (40); HRMS (EI) calcd for $C_{18}H_{19}NO_3$: 297.1365, found 297.1368.



***Nor*oripavine (36).**

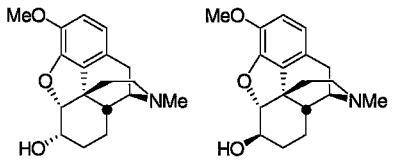
The title compound **36** was isolated following the protocol for biotransformations using *Cunninghamella echinulata*, ATCC 9244 in 9-21 % yield as colorless oil. Data for **36** are identical to those published in the literature.[95] R_f 0.15 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -69.39° (c = 0.475, MeOH); literature value is $[\alpha]_D$ = 90° (c = 0.27, MeOH)[103]; 1H NMR (MeOH-d_4, 600 MHz) δ: 6.66 (d, *J* = 8.2 Hz, 1H), 6.63 (d, *J* = 8.2 Hz, 1H), 5.94 (d, *J* = 6.4 Hz, 1H), 5.39 (s, 1H), 5.23 (d, *J* = 6.4 Hz, 1H), 4.57

(d, J = 6.8 Hz, 1H), 3.67 (s, 3H), 3.21 -3.38 (m, 6H), 2.25 - 2.36 (m, 1H), 2.02 (d, J = 13.5 Hz , 1H) ppm; ^{13}C NMR ($CDCl_3$, MeOH-d_4 150 MHz) δ: 154.5, 143.6, 140.1, 131.7, 124.7, 122.9, 119.7, 117.1, 116.9, 94.8, 87.5, 54.4, 53.6, 44.9, 37.2, 34.2, 33.3, ppm; MS (EI) *m/z* (%) 284 (17), 283 (55), 282 (29), 265 (15), 229 (16), 228 (13), 227 (10), 149 (23), 97 (11), 84 (11), 83 (15), 82 (11), 71 (17), 70 (15), 69 (18), 57 (30), 56 (13), 55 (23), 46 (20), 45 (100), 44 (45) ; HRMS (EI) calcd for $C_{17}H_{17}NO_3$: 283.1208, found 283.1214.



Noroxycodone (35).

The title compound **35** was isolated following the protocol for biotransformations using *Cunninghamella echinulata*, ATCC 9244 in 15 % yield as colorless oil in 80 % purity. Data for **35** matches closely to those published in the literature.[96] R_f 0.21 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -102.668° (c = 0.875, MeOH); literature value is $[\alpha]_D25$ = +100.4° (c = 0.55, MeOH)[101](enantiomer); IR (film) ν_{max}: 3399, 3350, 3007, 2932, 2838, 1725, 1636, 1608, 1505, 1440, 1277, 1051 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ: 6.73 (d, J = 8.2 Hz, 1H), 6.66 (d, J = 8.2 Hz, 1H), 4.68 (s, 1H), 3.92 (s, 3H), 2.95 – 3.28 (m, 3H), 2.68 -2.86 (m, 2H), 2.22 - 2.50 (m, 3H), 1.85 – 1.97 (m, 1H), 1.47 – 1.74 (m, 2H) ppm; MS (EI) *m/z* (%) 301 (5), 88 (11), 86 (65), 84 (100), 83 (12), 49 (18), 47 (23); HRMS (EI) calcd for $C_{17}H_{19}NO_4$: 301.1314, found 301.1316.

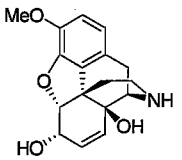


Dihydrocodeine (38) and *iso*-dihydrocodeine (41).

A mixture of compound **38** and **41** (ratio 5: 4) was isolated following the protocol for biotransformations using *Curvularia lunata,* ATCC 12017, in 40% combined yield as colorless oil. Careful purification by flash column chromatography ($CHCl_3$/MeOH) allowed for the preparation of analytical pure samples. Data for dihydrocodeine (**38**) are identical to those published in the literature.[97] R_f 0.55 (DCM : MeOH : NH_4OH, 98:2:1); ^1H NMR ($CDCl_3$, 600 MHz) δ: 6.74 (d, J = 8.1 Hz, 1H), 6.65 (d, J = 8.2 Hz, 1H), 4.62 (d, J = 5.7 Hz, 1H), 4.03 – 4.08 (m, 1H), 3.89 (s, 3H), 3.08 – 3.12 (m, 1H), 3.02 (d, J = 18.3 Hz 1H), 2.53 (dd, J = 12.0, 4.6 Hz 1H), 2.36- 2.44 (m, 4H), 2.20 - 2.29 (m, 2H), 1.90 (td, J = 12.3, 5.0 Hz, 1H), 1.69 – 1.75 (m, 1H), 1.55 - 1.62 (m, 1H) 1.41- 1.53 (m, 2H), 1.10 - 1.19 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 146.2, 141.6, 130.4, 127.0, 119.2, 113.1, 90.5, 67.2, 59.7, 56.4, 46.8, 43.0, 42.2, 40.7, 37.5, 27.2, 20.0, 19.2 ppm; MS (EI) *m/z* (%) 302 (20), 301 (100), 300 (23), 299 (32), 244 (14), 243 (11), 242 (19), 185 (12), 164 (16), 115 (10), 70 (19), 59 (24), 44 (23); HRMS (EI) calcd for $C_{18}H_{23}NO_3$:301.1678, found 301.1678.

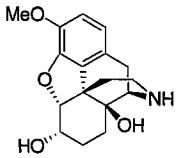
Data for *iso*-dihydrocodeine (**41**) are identical to those published in the literature[97]. R_f 0.51 (DCM : MeOH : NH_4OH, 98:2:1); ^1H NMR ($CDCl_3$, 600 MHz) δ: 6.73 (d, J = 8.2 Hz, 1H), 6.65 (d, J = 8.2 Hz, 1H), 4.37 (d, J = 6.5 Hz, 1H), 3.89 (s, 3H), 3.43 – 3.50 (m, 1H), 3.09 (m, 1H), 3.03 (d, J = 18.3 Hz, 1H), 2.53 (dd, J = 11.9, 4.5 Hz, 1H), 2.42 (s, 3H), 2.36 (dd, J = 18.3, 5.3 Hz, 1H), 2.12 – 1.23 (m, 2H), 1.80 - 1.90 (m, 2H) 1.69-

1.74 (m, 1H), 1.56 - 1.63 (m, 1H), 1.38 (q, J = 12.9 Hz, 1H), 1.00 (q, J = 12.9 Hz, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 144.1, 143.5, 130.5, 126.7, 119.0, 113.3, 97.2, 73.3, 59.4, 56.5, 47.0, 43.2, 43.0, 42.9, 35.6, 30.0, 23.7, 20.1 ppm; MS (EI) *m/z* (%) 302 (20), 301 (100), 300 (20), 299 (16), 286 (11), 244 (26), 242 (12), 185 (12), 86 (50), 84 (73), 70 (21), 59 (31), 57 (32), 56 (21), 55 (11), 49 (12), 47 (14), 44 (23); HRMS (EI) calcd for $C_{18}H_{23}NO_3$:301.1678, found 301.1672.



14-hydroxycodeine (40).

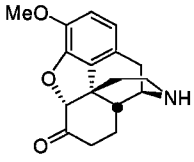
The title compound **40** was isolated following the protocol for biotransformations using ***Trametes sanguinea*** (ATCC 14622) and ***Trametes cinabarina*** (ATCC 14623) in 35-50% yield as colorless solid. mp 109 -111 °C (MeOH/diethyl ether); R_f 0.25 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -127.89° (c = 0.3, $CHCl_3$); $[\alpha]_D$ = -129.5° (c = 1.75, $CHCl_3$)[104] ; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 6.65 (d, J = 8.2 Hz, 1H), 6.57 (d, J = 8.2 Hz, 1H), 5.91 (d, J = 9.8 Hz, 1H), 5.49 (dd, J = 9.9, 2.9 Hz, 1H), 4.87 (d, J = 6.5 Hz, 1H), 4.60 – 4.65 (m, 1H), 3.83 (s, 3H), 3.18 (d, J = 18.5 Hz, 1H), 3.03 (d, J = 6.4 Hz, 1H), 2.98 (d, J = 10.2 Hz , 1H), 2.45 – 2.57 (m, 2H), 2.41 (s, 3H), 2.35 – 2.39 (m, 2H), 1.72 – 1.79 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 145.5, 142.5, 138.1, 132.3, 128.8, 125.8, 119.3, 113.1, 90.0, 68.7, 65.2, 64.0, 56.3, 46.7, 45.2, 42.8, 31.7, 22.2 ppm; MS (EI) *m/z* (%) 316 (20), 315 (100), 313 (12), 230 (19), 229 (31), 188 (16), 175 (11), 143 (31), 141 (12), 140 (21), 131 (14), 150 (12), 70 (56), 58 (17), 55 (11), 44 (58); HRMS (EI) calcd for $C_{18}H_{21}NO_4$: 315.1471, found 315.1470.

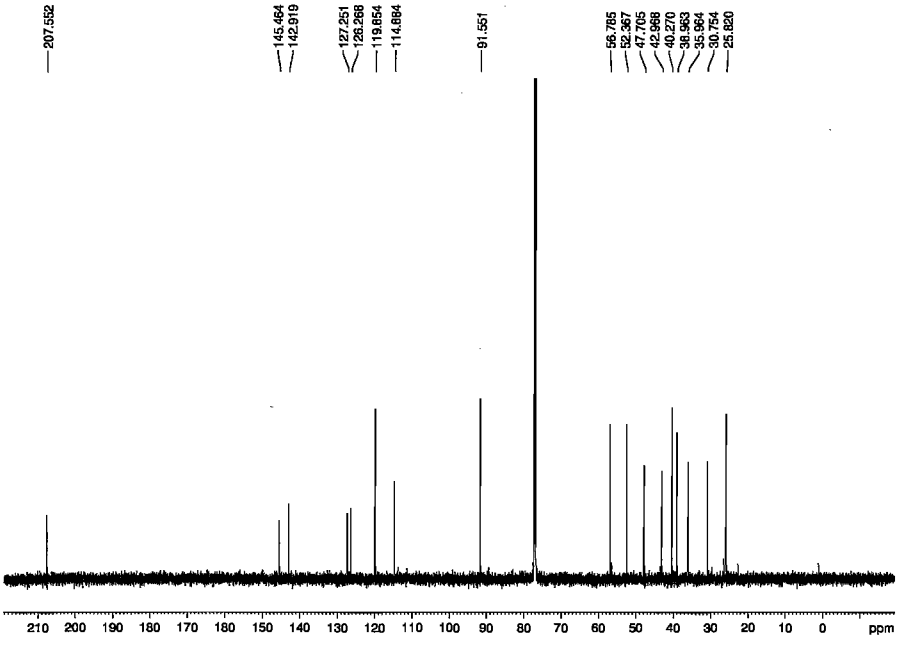


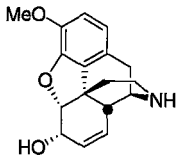
14-hydroxydihydrocodeine (39).

The title compound **39** was isolated following the protocol for biotransformations using ***Trametes sanguinea*** (ATCC 14622) in 10- 15% yield as colorless oil. Data (^{1}H NMR and ^{13}C NMR) for compound 39 were found to be identical to the major isomer obtained from the reduction of oxycodone with sodium borohydride in methanol. R_f 0.35 (DCM : MeOH : NH_4OH, 98:2:1); $[\alpha]_D20$ = -93.12° (c = 0.65, $CHCl_3$); literature values are $[\alpha]_D$ = -169° (c = 0.6, $CHCl_3$)[104], $[\alpha]_D$ = -138° (c = 1.8 in 10% acetic acid)[104]; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 6.78 (d, *J* = 8.2 Hz, 1H), 6.62 (d, *J* = 8.2 Hz, 1H), 4.78 (d, *J* = 6.5 Hz, 1H), 4.12 – 4.29 (m, 1H), 3.88 (s, 3H), 3.14 (d, *J* = 18.5 Hz, 1H), 2.80 (d, *J* = 5.5 Hz, 1H), 2.60 (dd, *J* = 18.5, 5.6 Hz , 1H), 2.40 – 2.51 (m, 1H), 2.38 (s, 3H), 2.19 – 2.31 (m, 2H), 1.74 – 1.89 (m, 1H), 1.38 – 1.70 (m, 3H), 1.09 – 1.28 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 146.5, 141.7, 131.4, 126.1, 118.9, 113.8, 90.8, 70.0, 66.7, 64.7, 56.5, 46.3, 45.0, 43.1, 33.2, 28.2, 23.7, 22.1 ppm.

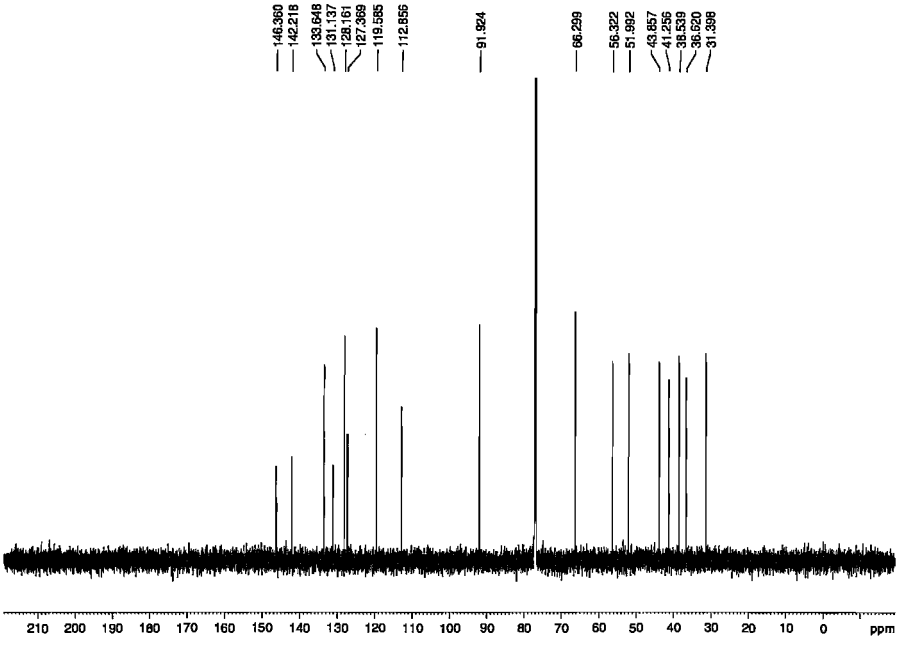
6. Selected spectra

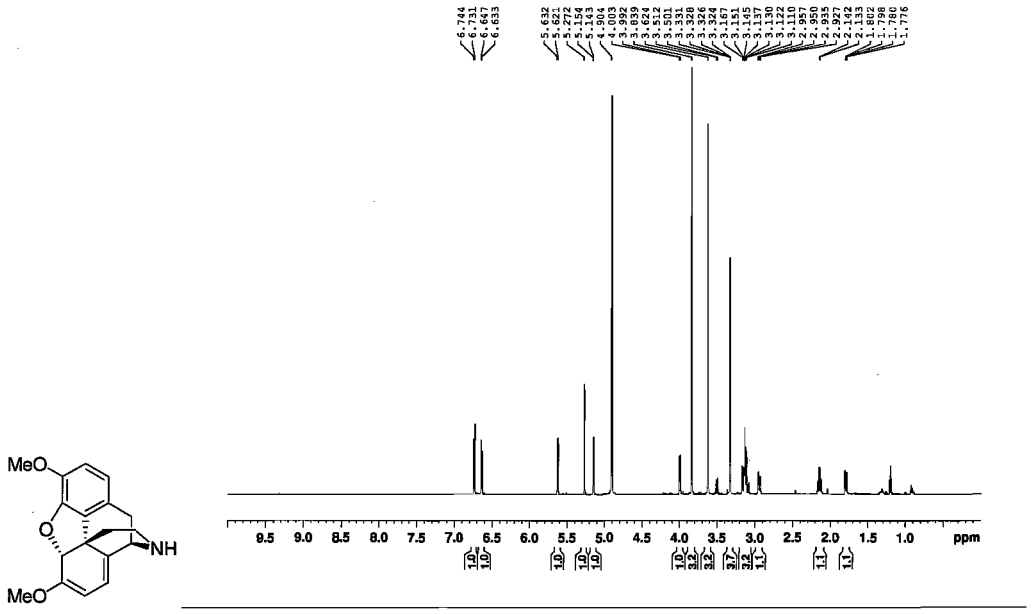





MeO
O
NH
HO

6.703
6.689
6.606
6.593
5.749
5.732
5.319
5.295
5.291
5.267
5.279
5.274
5.270
4.895
4.884
4.747
4.202
4.198
4.192
4.188
4.183
3.866
3.675
3.669
3.666
3.660
2.978
2.971
2.959
2.952
2.913
2.907
2.876
2.866
2.842
2.812
2.514
2.509
2.505
1.948
1.940
1.929
1.922
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.04
1.03
1.00
1.00
1.01
1.02
3.15
1.06
1.15
1.18
1.05
1.24
1.19
2.28


146.360
142.218
133.648
131.137
128.161
127.369
119.585
112.856
91.924
66.299
56.322
51.992
43.857
41.256
38.539
36.620
31.398
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm


MeO
O
NH
MeO
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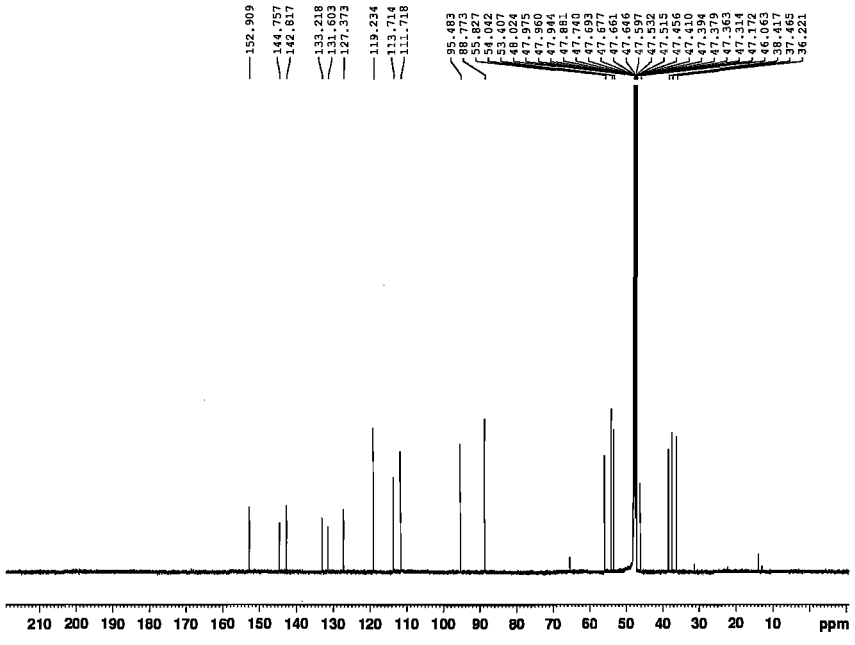
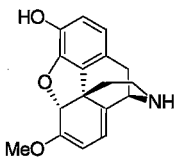
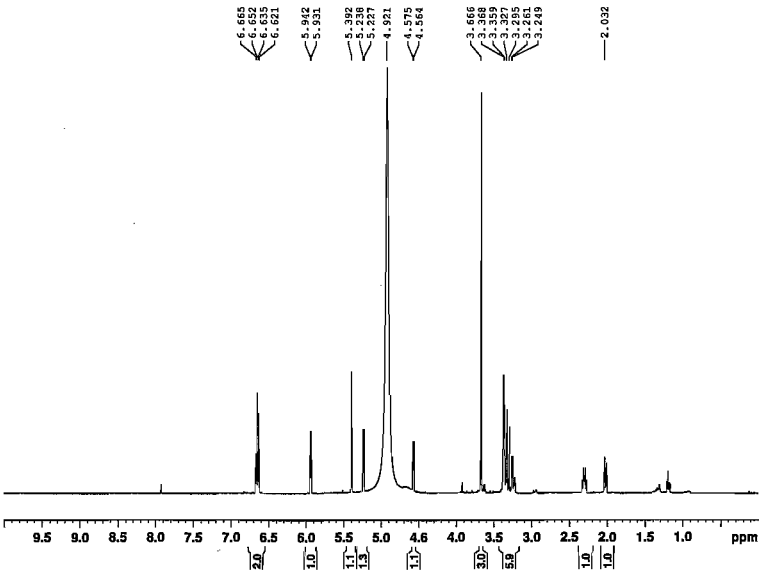
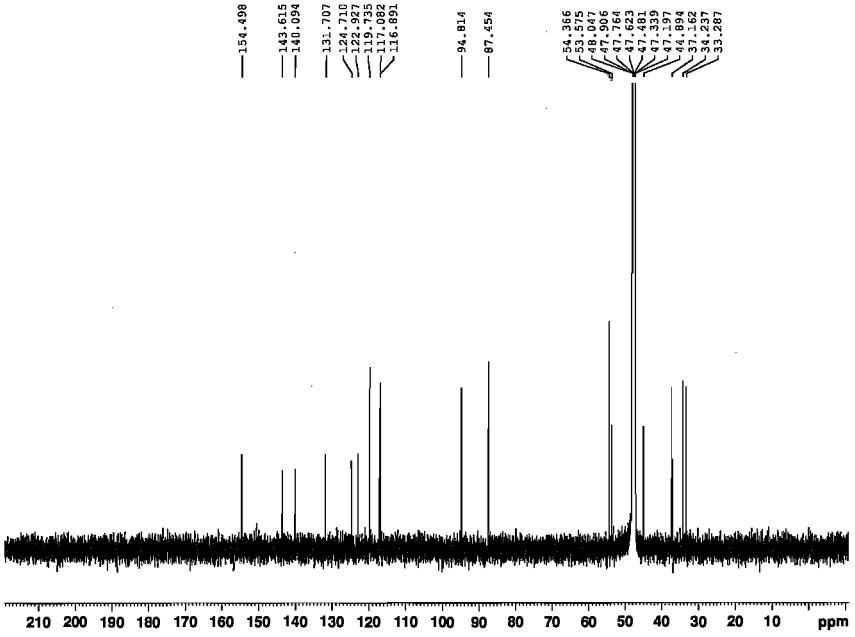
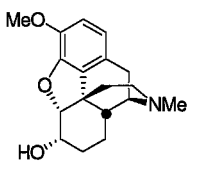
6.647
6.633
5.632
5.621
5.272
5.154
5.143
4.906
4.003
3.992
3.839
3.824
3.812
3.501
3.331
3.328
3.326
3.324
3.167
3.151
3.145
3.137
3.130
3.122
3.110
2.957
2.950
2.935
2.927
2.142
2.133
1.982
1.798
1.780
1.776
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.0 1.0 1.0 1.0 1.0 3.2 3.2 2.7 2.2 1.7 1.1 1.1

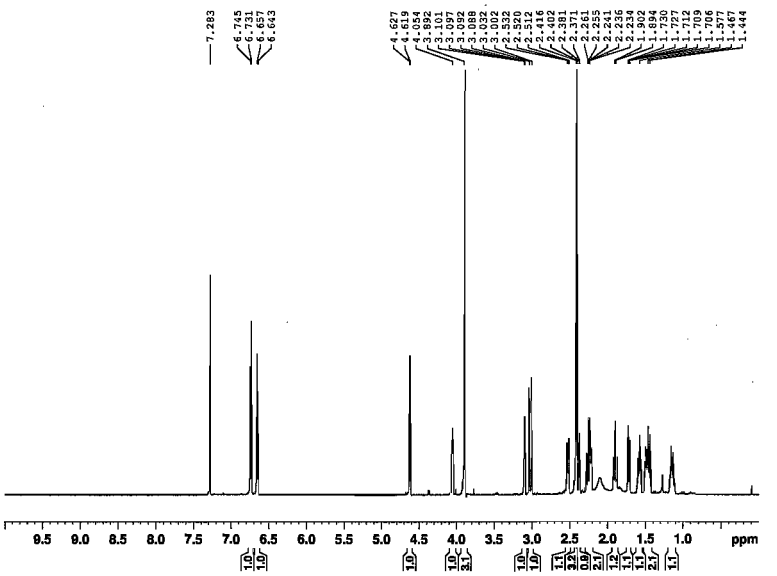

152.909
144.757
142.817
133.218
131.603
127.373
119.234
113.714
111.718
95.483
88.773
55.827
54.042
53.407
48.004
47.975
47.960
47.944
47.881
47.740
47.693
47.677
47.661
47.646
47.597
47.532
47.515
47.456
47.410
47.394
47.379
47.363
47.314
47.272
46.063
38.417
37.465
36.221
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

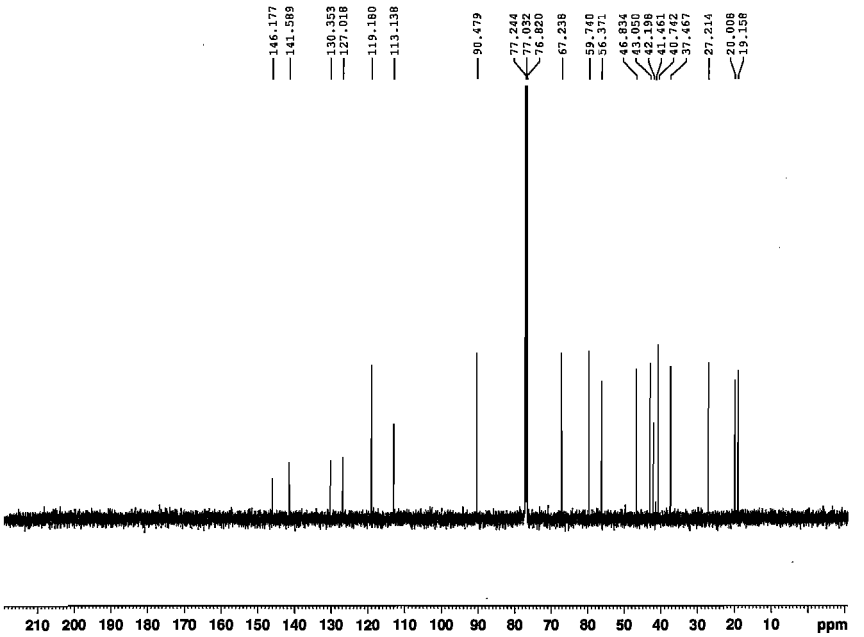

HO
O
NH
MeO


6.665
6.652
6.635
6.621
5.942
5.931
5.392
5.236
5.227
4.921
4.575
4.564
3.666
3.368
3.359
3.327
3.295
3.261
3.249
2.032
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.6 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
2.0
1.0
1.1
1.3
1.1
3.0
5.8
1.0
1.0


154.498
143.615
140.094
131.707
124.710
122.927
119.735
117.082
116.891
94.814
87.454
54.366
53.575
48.047
47.906
47.764
47.623
47.481
47.339
47.197
47.056
46.914
37.162
34.237
33.287
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm


MeO
O
NMe
HO


7.283
6.745
6.731
6.657
6.643
4.627
4.619
4.054
3.892
3.101
3.097
3.092
3.088
3.032
3.002
2.983
2.520
2.512
2.416
2.402
2.381
2.371
2.261
2.255
2.241
2.236
2.234
1.902
1.694
1.730
1.727
1.714
1.709
1.706
1.577
1.467
1.444
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm


146.177
141.589
130.353
127.018
119.160
113.138
90.479
77.244
77.032
76.820
67.238
59.740
56.371
46.834
43.050
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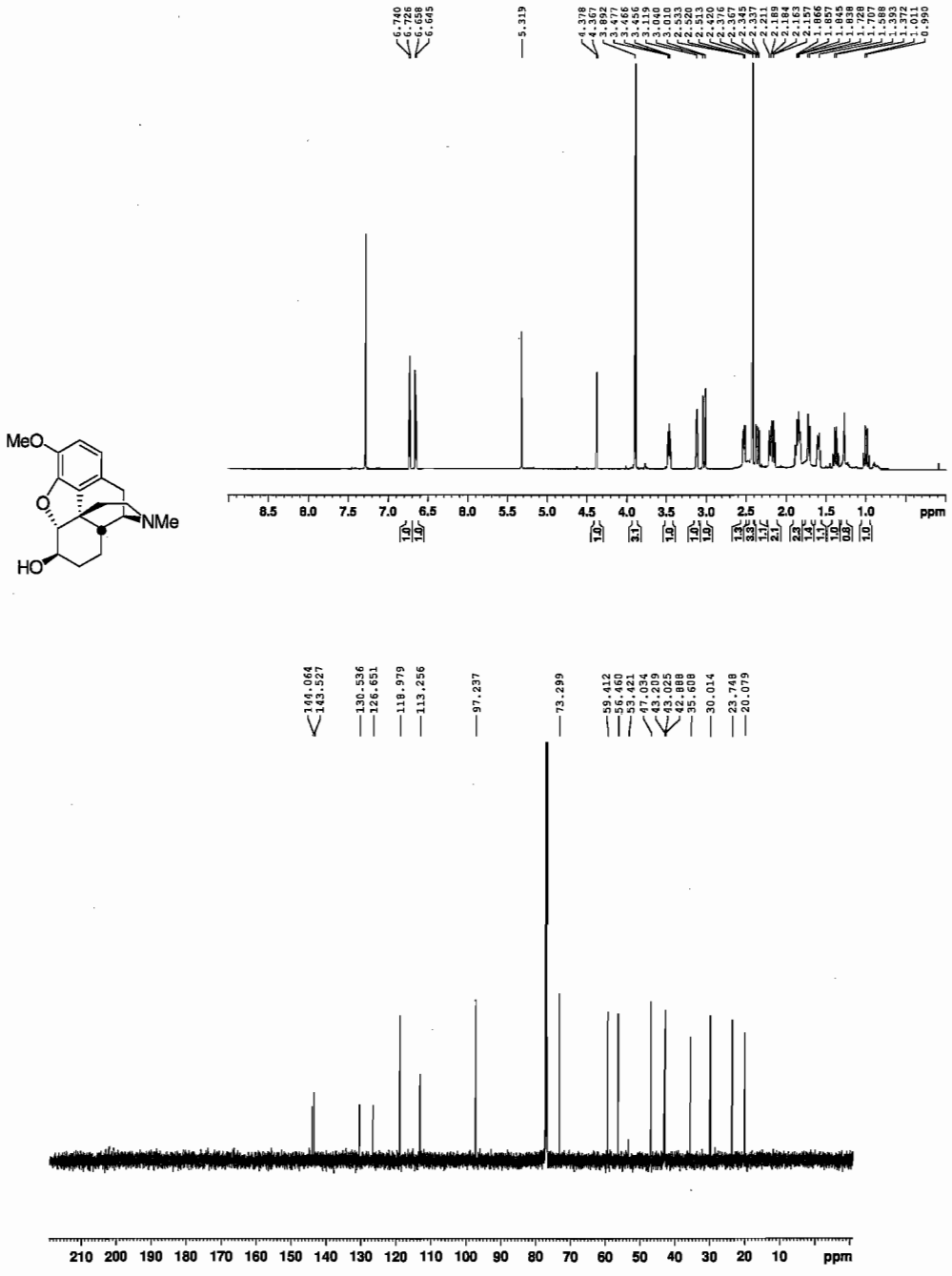
37.467
27.214
20.008
19.158
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 80 50 40 30 20 10 ppm


MeO
O
NMe
HO
6.740
6.726
6.658
6.645
5.319
4.378
4.367
3.892
3.477
3.466
3.456
3.119
3.040
3.010
2.553
2.520
2.513
2.420
2.376
2.367
2.345
2.337
2.211
2.189
2.184
2.163
2.157
1.866
1.857
1.846
1.839
1.728
1.707
1.588
1.393
1.372
1.011
0.990
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
144.064
143.527
130.536
126.651
118.979
113.256
97.237
73.299
59.412
56.460
53.421
47.034
43.209
43.025
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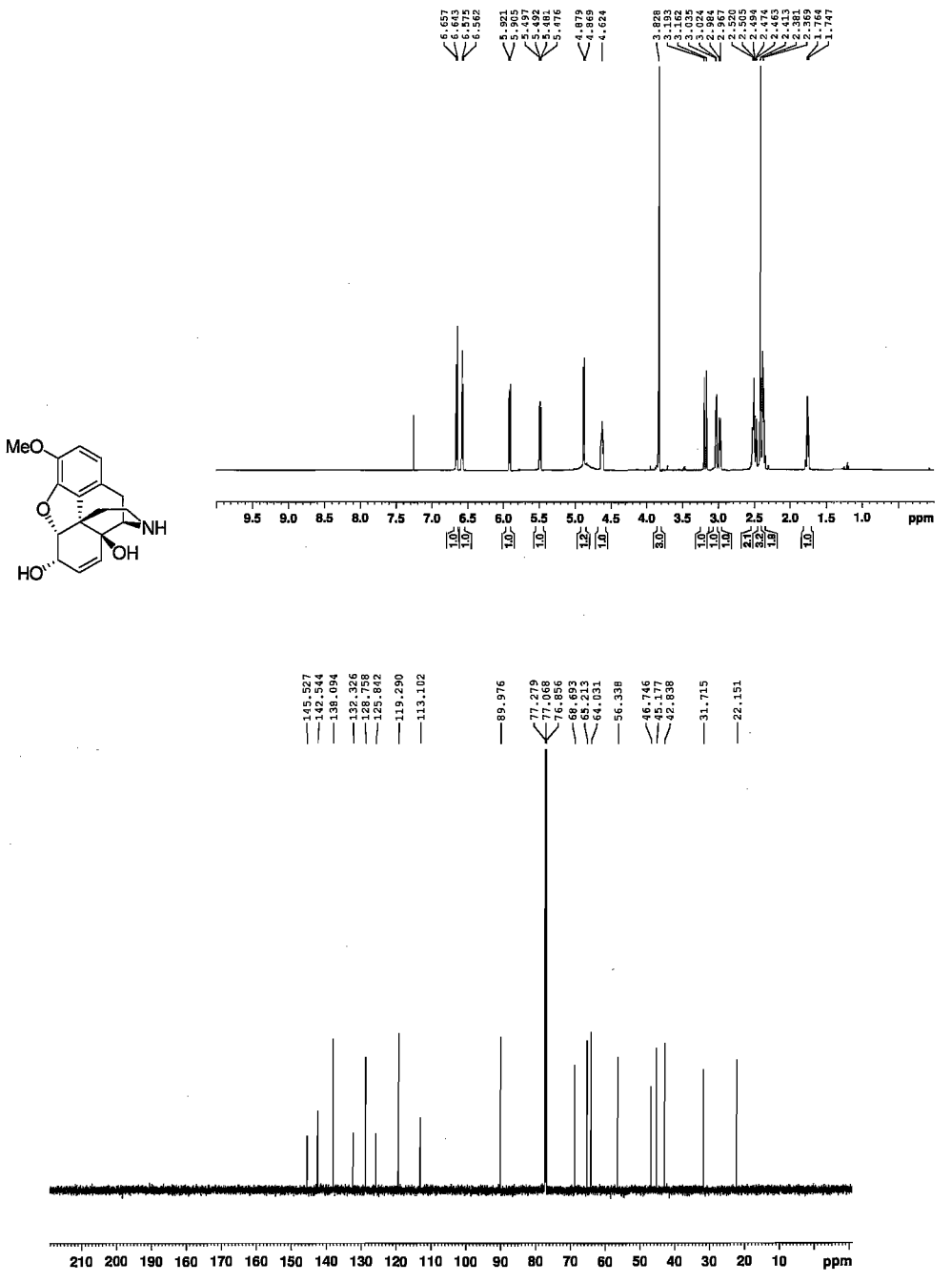
23.748
20.079
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm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HO
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8. Appendix 1

Table 1: Attempts to develop an enzyme assay using Method 1. Depending on the assay being conducted, they contained 0.5 mg crude protein extract, Thebaine, NADPH or α-Ketoglutarate. Enzyme assays lasted for 1 h at 37 °C. Enzyme assays conducted with denatured protein (B) were performed by boiling extracts for 10 min before assay. At the end of the enzyme assay, samples were extracted for alkaloids as described in Materials and Methods.

Sample	Protein	NADPH	Thebaine	α-Ketoglutarate
1	+	+	+	-
2	+	-	+	-
3	+ B	+	+	-
4	+	-	+	2mM
5	+ B	-	+	2mM

'+' indicates the presence of the substance and '-'indicates the absence of the substance in the enzyme assays.

Table 2: Attempts to develop an enzyme assay using Method 2. Depending on the assay being conducted, they contained 0.76 mg crude protein extract, Thebaine, NADPH, α-Ketoglutarate, Ferrous ions, Ascorbic acid, Flavin adenine mononucleotite (FMN) or Flavin adenine dinucleotide (FAD). Enzyme assays lasted for 1 h at 37 °C. Enzyme assays conducted with denatured protein were performed by boiling extracts for 10 min before assay. At the end of the enzyme assay, samples were extracted for alkaloids as described in Materials and Methods.

Enzyme Assay	1	2	3	4	5	6	7	8
Protein (500 µL)	+	+B	+	+B	+	+B	+	+B
Thebaine (50 µg)	+	+	+	+	+	+	+	+
NADPH (1 mM)	+	+	-	-	-	-	+	+
NADH (1 mM)	-	-	+	+	-	-	-	-
α-keto glutarate (2 mM)	-	-	-	-	+	+	-	-
Ferrous (1 mM)	-	-	-	-	+	+	-	-
Ascorbic acid (250 mM)	-	-	-	-	+	+	-	-
FMN (1 mM)	-	-	-	-	-	-	+	+
FAD (1 mM)	-	-	-	-	-	-	+	+

'+' indicates the presence of the substance and '-'indicates the absence of the substance in the enzyme assays.

Table 3: Enzyme assays using higher (approximately double compared to table 2 assays) concentration of thebaine. Two controls were used in which one did not have any co-factors and the other did not have the substrate thebaine in order to avoid false positive reactions. Stock solution was prepared by making a saturated solution (1.5 mg thebaine in 40 µL DMSO) of thebaine in DMSO. Concentration of total proteins was 1.4 mg/mL.

Sample	1	2	3	4	5	6	7	8	9	10	11	12
Protein (500 µL)	+	+	+	+	+	+	+	+	+	+	+	+
Thebaine (5 µL from stock)	+	+	+	+	+	+	+	+	+	+	+	-
NADPH (1 mM)	+	+	-	-	-	-	+	+	-	-	-	-
NADH (1 mM)	-	-	+	+	-	-	-	-	-	-	-	-
α-keto glutarate (2 mM)	-	-	-	-	+	+	-	-	-	-	-	-
Ferrous (1 mM)	-	-	-	-	+	+	-	-	-	-	-	-
Ascorbic acid (250 mM)	-	-	-	-	+	+	-	-	-	-	-	-
FMN (1 mM)	-	-	-	-	-	-	+	+	-	-	-	-
FAD (1 mM)	-	-	-	-	-	-	+	+	-	-	-	-
AdoMet (1 mM)	-	-	-	-	-	-	-	-	+	+	-	-
AdoCys (1 mM)	-	-	-	-	-	-	-	-	+	+	-	-

'+' indicates the presence of the substance and '-'indicates the absence of the substance in the enzyme assays.

In the above experiment the cells were first ground using mortar and pestle and the homogenate was subjected to sonication. The sonication buffer contained 1mM DTT

(dithiothreitol), 14 mM β-mercaptoethanol and 50mM Tris HCl buffer at pH-7.5. After sonication, the crude extract was filtered through a layer of mira cloth and was centrifuged at 500g for 10 minutes at 4 °C. The supernatant was desalted through PD10 column and the filtrate was used for enzyme assays. The incubation of enzyme was done at 37 °C with the substrate and co-factors for one hour.

Table 4: Enzyme assays using Gibson's procedure. Concentration of total proteins was 3.75 mg/mL before desalting and 2.6 mg/mL after desalting through PD 10 column.

Sample	Protein 1 mL	Thebaine 100 µg	NADPH 1 mM	NADH 1 mM	Ferrous 5 mM	Semicarbazide-HCl 30 mM	Glycine 10 mM
1)	+(not desalted)	+	+	+	+	+	-
2)	+ (desalted)	+	+	+	+	+	-
3)	+ (desalted)	+	+	+	+	-	+
4)	+ (desalted)	+	-	-	-	-	-
5)	+ (desalted)	-	-	-	-	-	-

'+' indicates the presence of the substance and '-'indicates the absence of the substance in the enzyme assays.

Table 5: 50 mM Tris buffer at pH- 7.5 was used to extract fungal proteins.

Sample	Protein 2 mL	Thebaine 200 µg	NADPH 1 mM	NADH 1 mM	Ferrous 5 mM	Semicarbazide-HCl 30 mM
1)	+	+	+	+	+	+
2)	+	+	-	-	-	-
3)	+	-	-	-	-	-

'+' indicates the presence of the substance and '-'indicates the absence of the substance in the enzyme assays.

The above set of experiments (table 6) were repeated separately by using 66 mM phosphate buffer with pH- 7.0 and 66 mM phosphate buffer with pH- 7.5 as extraction buffers for protein extraction from fungal cells. Concentration of total proteins in Tris buffer was 1 mg/ mL, in phosphate buffer pH- 7.0 was 1.5 mg/mL and in phosphate buffer at pH- 7.5 was 1.2 mg/mL. But none of the experiments provided a positive result.

9. Vita

Vigi George Chaudhary was born in Kerala, India on 20th December 1977. She and her brother, Aji George, were raised by their parents, Elizabeth and George. She attended elementary school at Puthencruz, and high school at Kochi, in Kerala. As a child her passion was playing chess. She also liked badminton and Bharatnatyam. After graduation from high school in 1996, she went to Kannur University, Kerala to study Microbiology. She was college chess champion in 1998. After finishing undergraduate degree with distinction, she joined Calicut University, Kerala to pursue Master's in Microbiology under the supervision of Prof. Ignatius D. Konikkara. She also got the opportunity for training in molecular biology techniques at the Rajiv Gandhi Centre for Biotechnology, Kerala during her Master's program. After successfully completing her Master's in Microbiology, she moved to Jaipur, to teach Microbiology at a Dental School in 2002. In 2006, she was selected for Canadian Commonwealth Scholarship and she moved to St. Catharines, Canada to pursue Master's in Biotechnology under the supervision of Prof. Tomas Hudlicky at Brock University. She is presently working towards completion of her Master's degree in Chemical Biotechnology. Her research interests include screening of microbial species capable of industrially relevant biotransformations.